Multiple Binding Modes of the Single-Stranded DNA Binding Protein from *Escherichia coli* As Detected by Tryptophan Fluorescence and Site-Directed Mutagenesis†

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ABSTRACT: We have systematically substituted the four tryptophan residues of the single-stranded DNA binding protein from *Escherichia coli* (EcoSSB) by polar (serine or threonine) and aromatic (tyrosine or phenylalanine) amino acids. The resulting mutants with either single amino acid exchanges or triple substitutions are all active in ssDNA binding, though in some cases with reduced affinities. Measurements of the fluorescence of the mutated EcoSSBs show that there is no interaction between the four different tryptophan residues. We analyzed the ssDNA binding of the mutant proteins by fluorescence titrations. At 0.3 M NaCl ("high salt"), all singly substituted proteins bind to poly(dT) in a manner comparable to wild-type EcoSSB, covering 65 nucleotides with 1 EcoSSB tetramer. W54S mutant protein is an exception since even at 0.3 M NaCl it covers approximately 35 nucleotides, a behavior which is typical of salt concentrations below 10 mM NaCl ("low salt"). From this observation, it is inferred that tryptophan-54 is involved in a direct interaction with the ssDNA favoring the "high-salt" binding mode. All mutant proteins lacking tryptophan-54 but possessing tryptophan-88 at "low-salt" concentrations show a nonmonotonous behavior in the fluorescence titrations. This behavior can be interpreted assuming a model of cooperative binding of EcoSSB to poly(dT) with two different binding site sizes ($n \approx 27$ and $n \approx 33$) and different binding affinities. A quantitative treatment of the problem of multiple binding modes in the interaction of a multidentate ligand with a linear polymer is applied to these titrations.

The single-stranded DNA (ssDNA) binding protein of *Escherichia coli* (EcoSSB)[1] plays a vital role in the metabolism of bacterial DNA. The protein binds strongly and cooperatively to ssDNA and has very low affinity to double-stranded DNA. EcoSSB protects the ssDNA against degradation and prevents intramolecular hairpin formation, thereby, for example, enhancing the DNA polymerase activity or catalyzing the polymerization of recA protein in homologous recombination. The protein has a molecular mass of 18 873 daltons and forms a tetramer under almost all conditions. Each protomer contains four tryptophan, four tyrosine, and four phenylalanine residues. For recent reviews of EcoSSB functions, cf. Chase and Williams (1986), Greipel et al. (1989), Lohman and Bujalowski (1990), and Meyer and Laine (1990).

Up to now, EcoSSB withstood all attempts to determine its tertiary structure. The protein had been crystallized, but these crystals contained proteolytic fragments (Ollis et al., 1983; Monzingo & Christiansen, 1983; Hilgenfeld et al., 1984; Ng & McPherson, 1989). A D_2 symmetry had been deduced from preliminary X-ray analysis of these crystals (Ollis et al., 1983), but no detailed high-resolution X-ray structure has been obtained yet.

Thus, to obtain structure–function relationships for this protein, one can resort to studies in solution. One of the most important features to be investigated is the binding of the protein to ssDNA. This binding conveniently can be followed by monitoring the intrinsic tryptophan fluorescence of the protein, which is reduced by up to 90% when the ssDNA is bound. There have been a large number of investigations of the binding of EcoSSB to various ssDNAs under various conditions [for a recent review, cf. Lohman and Bujalowski (1990)].

The binding properties of EcoSSB reported in these studies show a pronounced salt dependence. Below 10 mM NaCl ("low salt"), 35 dT residues of poly(dT)[1] are covered by 1 EcoSSB tetramer whereas above 200 mM NaCl ("high salt") 65 nucleotides are bound. A model has been proposed where at "low salt" only two of the four subunits of the tetramer are used while at "high-salt" concentrations all four protomers are covered by poly(dT) (Lohman et al., 1986a).

To elucidate the role of certain amino acids in the structure and function of the protein, site-directed mutagenesis can be combined with binding studies. We have applied this technique to further characterize the role of the amino acids at positions 60 (Bayer et al., 1989) and 55 (Curth et al., 1991) in the structure and function of EcoSSB. These amino acids are involved in a hydrophobic interaction with ssDNA (F60) and the tetramerization of the protein (H55). In the present work, we will use site-directed mutagenesis to investigate the role of the four tryptophan residues of the protein in positions 40, 54, 88, and 135.

MATERIALS AND METHODS

Poly(dT) was purchased from Pharmacia (Freiburg, FRG). It had an average length of 1400 bases as judged from hydrodynamic measurements and gel electrophoresis. Concentrations of poly(dT) were determined using an extinction coefficient of 8600 M^{-1} cm^{-1} at maximum (Urbanke & Schaper, 1990) and are given in monomer units throughout the text.

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[1] Abbreviations: poly(dT), poly(thymidylic acid); EcoSSB, single-stranded DNA binding protein from *Escherichia coli*; ssDNA, single-stranded DNA; KP_i, potassium phosphate.

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Measurements were performed in a standard buffer containing 20 mM potassium phosphate, pH 7.4 (unless specified otherwise), 0.1 mM EDTA, 100 ppm of TWEEN 20 (Serva), and different concentrations of NaCl as indicated.

Mutagenesis Procedures. The construction of the EcoSSB-overproducing plasmid pSF1 has been described earlier (Bayer et al., 1989). Since this vector contains the replication origin of f1 phage, it can be used for production of single-stranded DNA required for mismatch primer mutagenesis.

General Strategies for Construction of Mutant EcoSSB. All mutations were introduced using mismatch primed DNA synthesis on gapped duplex substrates (Geiger et al., 1989; Fritz et al., 1988). Generally, mutant plasmids contained an additional restriction site to facilitate screening. Oligonucleotides were synthesized on a Cyclone DNA synthesizer (Milligen) using the β-cyanoethyl phosphoamidite technique. After deprotection, they were used for mutagenesis without further purification. For the triple exchange of tryptophan residues, suitable restriction fragments containing one or two mutations were combined.

Single amino acid exchanges are designated by their respective one-letter code, e.g., W88T being a tryptophan at position 88 replaced by threonine. Triple mutants are designated by the respective three-letter code with Trp88 being a protein combining the W40T, W54S, and W135T mutants (Trp40: W54S, W88T, and W135T; Trp54: W40T, W88T, and W135T; Trp135: W40T, W54S, and W88T).

DNA Sequencing. DNA sequencing using T7 polymerase (Pharmacia, Freiburg, or AGS, Heidelburg) was done according to the standard protocol provided by Pharmacia using double-stranded plasmid DNA as template. For each mutation, the complete ssb gene was sequenced. No changes except the desired mutation were detected.

Protein Preparation. Mutant EcoSSB proteins were prepared according to the method described by Lohman et al. (1986b) for wild-type EcoSSB.

Extinction coefficients for the mutant proteins were calculated from the known extinction coefficients of the aromatic amino acids (Bredderman, 1974) and the known composition of the protein. For the wild-type and W54S, W54F, W88T, W88Y, W40T, Trp54, and W40Y mutant proteins, we determined the extinction coefficient using the second-derivative absorption spectrum (Levine & Federici, 1982) and found no difference from the calculated value. Protein concentrations are given in units of tetramers throughout the text.

Analytical ultracentrifugation was performed in a Spinco/Beckman Model E centrifuge equipped with an UV scanner and interfaced to a personal computer for data acquisition and evaluation. NMR spectra were taken in a Bruker WH270 spectrometer as described previously (Bayer et al., 1989). Stopped-flow experiments were performed in a modified version of a Durrum-Gibson stopped-flow apparatus as described by Urbanke and Schaper (1990).

Mobility shift assays were performed in a native agarose gel as described by Lohman et al. (1986a). M13mp8 ssDNA was stained with ethidium bromide, poly(dT) with Stains All (purchased from Aldrich), and protein with Coomassie blue.

Fluorescence measurements were carried out in a Schoeffel RRS1000 spectrofluorometer. The relative fluorescence intensities for the different mutant proteins were determined from the initial slope of the concentration dependence of the fluorescence intensity with wild-type protein arbitrarily set to 100%. Excitation was at 295 nm, and emission was observed at 350 nm.

Two types of fluorescence titrations were used in this work. In "normal" titrations, protein was added to poly(dT). In these experiments, a fluorescence quench due to binding is expressed as a diminished slope of the titration curve. In "inverse" titrations, a mixture of protein and poly(dT) was added to the protein such that at all points the protein concentration was kept constant. Inverse titrations often are evaluated with respect to an "apparent binding site size" by using the intersection of the limiting slopes at low and high ssDNA/protein ratios. In both types of titration, the absorbance of the solution was below 0.05 at the excitation wavelength of 295 nm to avoid inner filter effects. Emission was observed at 350 nm. After each addition, the solution was allowed to equilibrate between 60 and 600 s until no fluorescence change could be observed any longer.

Evaluation of Binding Parameters. EcoSSB has four binding sites for nucleic acids, and the number of nucleotides covered by a single protein strongly depends on the conditions. The theoretical treatment of the binding of such a multidentate ligand to a linear polymer has been described in detail by McGhee and vonHippel (1974) and Schwarz and Watanabe (1983). Both treatments, although different in their resulting equations, yield identical numerical results. We have programmed the algorithm of Schwarz and Watanabe to be used in a fitting procedure (VA05A; Harwell Library, Oxfordshire, U.K.) to evaluate binding parameters from fluorescence titrations on a PC under MS-DOS. The program is capable of fitting binding parameters [stoichiometry (n), binding constant (K_{assoc}), cooperativity parameter (ω), and fluorescence of the complex (f^c) relative to the fluorescence of the respective free protein] to a complete set of titrations at different concentrations of reactants simultaneously.

If the ligand can bind in more than one binding mode, this treatment has to be extended. A general solution to this problem of multiple binding equilibria in the interaction of multidentate, interacting ligands to a large, linear polymer has been given by Y.-D. Chen (1990).

Programming this algorithm to calculate the binding isotherm requires finding of the largest root λ_1 of an nth-grade polynomial with $n-1$ being the sum of the binding site sizes of all binding modes (typically of the order of 100). This procedure can be facilitated using an argument of Schwarz and Stankowski (1979) where they give an estimate for the lower boundary of this root. For simplification, we always assumed that the cooperativity parameter between different binding modes is equal to 1. The program was combined with the fitting procedure VA05A as described above.

When fitting theoretical curves to experimental data, one has to be very cautious with respect to the significance of the results. We have tested both algorithms thoroughly with a wide range of parameter values. For the model of Schwarz and Watanabe, we found that the cooperativity parameter and the binding constant cannot be determined with great accuracy. Especially when high affinities are involved and the concentration of protein is not low enough, the affinity parameters (K_{assoc} and ω) can only be given as a lower estimate of the cooperative binding constant $K_{assoc}\cdot\omega$ while the stoichiometry n can be determined accurately. For the determinations of n and estimations of $K_{assoc}\cdot\omega$, we therefore used a simultaneous fit of several titrations done at different protein concentrations.

RESULTS AND DISCUSSION

We have replaced all four tryptophan residues of the EcoSSB either as a single mutation or as a triple mutation where all

Table I: Relative Fluorescence[a] of Tryptophan Mutants of EcoSSB (Wild Type = 100%)

mutants lacking one tryptophan	F(rel) (%)	100% − F(rel) (%)
W40T	91	9
W40Y	91	
W54S	54	45
W54F	56	
W88T	70	30
W88Y	70	
W135T	87	13
W135Y	87	
		$\Sigma = 97$

mutants containing only one tryptophan	F(rel) (%)
W54S/W88T/W135T ("Trp40")	10
W40T/W88T/W135T ("Trp54")	51
W40T/W54S/W135T ("Trp88")	33
W40T/W54S/W88T ("Trp135")	13
N-acetyltryptophan ethyl ester	6.3

[a] Relative fluorescence was measured at 295-nm excitation and 350-nm emission wavelengths (cf. Materials and Methods).

but one Trp residue have been substituted by threonine or tyrosine (serine or phenylalanine for tryptophan-54).

All proteins sedimented in the analytical ultracentrifuge with sedimentation rates comparable to wild-type protein, and we thus conclude that they essentially retained their tetrameric structure. We tested the structural integrity of the mutant proteins in detail by comparing their one-dimensional NMR spectra (270 MHz) as described for other mutant EcoSSBs (Bayer et al., 1989; Curth et al., 1991). No significant differences as compared to the wild-type structure were found.

Table I shows the fluorescence properties of the mutant proteins as compared to wild-type EcoSSB and free *N*-acetyltryptophan ethyl ester. The changes in fluorescence introduced by the replacement of single tryptophan residues add to nearly 100%. Furthermore, these changes are approximately equal to the fluorescence intensities of the corresponding triple mutant proteins where only one tryptophan residue is present. Thus, it is evident from both the singly mutated proteins as well as from the triple mutants that the fluorescence intensities of the four tryptophan residues are independent of each other and are not influenced by the mutations. There are large differences in the environment of the tryptophan residues as can be seen from the differences in their relative fluorescence intensities. The most intensive fluorescence originates from tryptophan-54 which contributes approximately 50% to the wild-type fluorescence whereas tryptophan-135 and tryptophan-40 have a fluorescence similar to that of free *N*-acetyltryptophan ethyl ester. We conclude that the latter tryptophan residues are exposed to the solvent and do not receive excitation energy from other aromatic amino acids.

In contrast to the large differences in fluorescence intensity, the spectral shapes of the emission spectra of wild-type and all mutant EcoSSB proteins are virtually identical. From the excitation and emission spectra of wild-type EcoSSB, it was concluded previously that the excited-state energy of tyrosines is either completely quenched or quantitatively transferred to tryptophan (Bandyopadhyay & Wu, 1978). The shapes and positions of the excitation spectra of all our EcoSSB proteins are also virtually identical although the absorption spectra are changed toward tyrosine absorption when tryptophans are removed. This indicates that the fluorescence of EcoSSB results from direct absorption of energy by the tryptophans

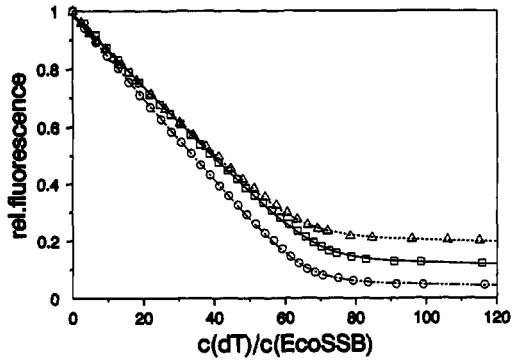

FIGURE 1: Inverse fluorescence titrations of 0.28 μM W40T (□), 0.37 μM W88T (△), and 0.28 μM W135T (○) EcoSSB with poly(dT) at 0.3 M NaCl. The solid lines show the best fit using a single binding mode: $n = 68$ and $f^c = 0.10$ (W40T); $n = 64$ and $f^c = 0.18$ (W88T); $n = 65$ and $f^c = 0.03$ (W135T). For binding constants and cooperativity parameters, cf. the text.

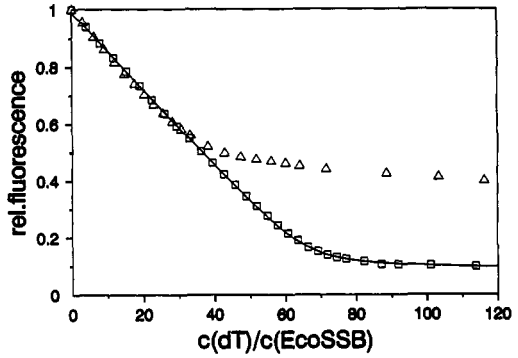

FIGURE 2: Inverse fluorescence titrations of 0.44 μM wild-type EcoSSB with poly(dT) at 0.3 M NaCl ("high salt") (□) and 1 mM NaCl/1 mM KP_i, pH 7.4 ("low salt") (△). The solid line represents the best fit using a single binding mode with $n = 66$ and $f^c = 0.08$. The "low-salt" titration cannot be fitted adequately by the simple model with one binding mode.

themselves and energy-transfer processes involving excited tyrosines do not contribute significantly.

Fluorometric monitoring of EcoSSB binding to ssDNA is an indirect method. Especially for mutant proteins containing only one tryptophan residue, it is important to demonstrate by independent methods that these mutants interact with ssDNA at all. For two mutants, we used analytical ultracentrifugation (Schaper et al., 1991) and for all four triple mutants mobility shift assays (Lohman et al., 1986a) as independent methods to detect binding. In analytical ultracentrifugation at 0.3 M NaCl, Trp54 EcoSSB formed a stable stoichiometric complex with poly(dT) while for Trp135 EcoSSB only weak binding could be detected (data not shown). Mobility shift assays of mixtures of protein and DNA incubated at 0.3 M NaCl in standard buffer and at 5 mM NaCl in 5 mM KP_i, pH 7.4, showed that all mutants could bind to M13mp8 ssDNA and poly(dT). A quantitative interpretation of these mobility shift assays with respect to thermodynamic parameters is not possible, since the ionic conditions of the gel are different from those of the incubation mixture and rearrangements of complexes during electrophoresis have to be considered.

At 0.3 M NaCl fluorescence titrations, W40T, W88T, and W135T mutant EcoSSBs did not show detectable alterations of binding to poly(dT) as compared to wild-type EcoSSB (for a typical result, cf. Figures 1 and 2). In fluorescence titrations, mutant proteins with tryptophan being replaced by the aromatic residue tyrosine (W40Y, W88Y, and W135Y) also showed no differences in poly(dT) binding as compared to the

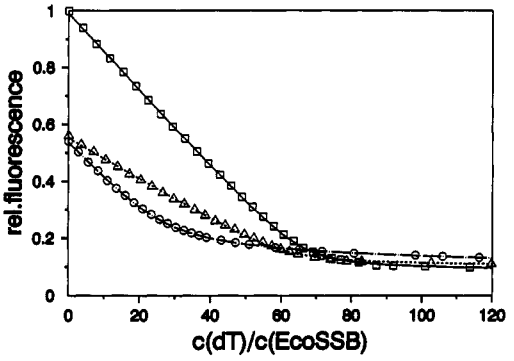

FIGURE 3: Inverse fluorescence titrations of 0.44 μM wild-type (□), 0.37 μM W54F (△), and 0.57 μM W54S (○) EcoSSB with poly(dT) at 0.3 M NaCl. The solid lines represent the best fit using a single binding mode with $n = 66$ and $f^c = 0.08$ (wild type), $n = 62$ and $f^c = 0.18$ (W54F), and $n = 34$ and $f^c = 0.13$ (W54S). Note that these f^c values refer to the fluorescence of the respective free mutant protein whereas the ordinate values are given relative to the fluorescence of free wild-type protein. For the fits, titrations at protein concentrations between 0.11 and 1.1 μM (W54F) and between 0.4 and 1.0 μM (W54S) were used.

respective threonine mutants and wild-type EcoSSB (data not shown). These titrations were done in the range of 50–370 nM protein, where only "stoichiometric" titration curves are observed. Assuming a cooperativity parameter ω between 500 and 50 (Greipel et al., 1987), all titrations could be fitted with a cooperative binding constant ($K_{assoc} \cdot \omega$) larger than 1×10^8 M^{-1}. A more accurate analysis of these "stoichiometric" fluorescence titrations with respect to binding constants and cooperativity parameters was not possible, since the concentrations that had to be used in the fluorescence titrations are too high and allow only a reliable determination of the binding site size n and a minimum estimate of the cooperative binding constant $K_{assoc} \cdot \omega$. Thus, while the binding site size n is unaffected by the mutations, only a drastic reduction of affinity would have been detectable.

The fluorescence of the W135T mutant protein is reduced by 97% when bound to poly(dT) at 0.3 M NaCl (cf. Figure 1). Since the fluorescence quench for wild-type EcoSSB is 92% under the same conditions, we conclude that the fluorescence of the most intensive fluorescing residues W88 and W54 is reduced almost completely by binding to poly(dT).

Mutations of the tryptophan residue at position 54 have a more drastic effect on the binding properties of the protein. At 0.3 M NaCl, wild-type and W54F EcoSSBs bind to poly(dT) with a stoichiometry n of 65 and 62, respectively (Figure 3), characteristic of a binding mode at "high-salt" concentrations as described by Lohman and Overman (1985). However, W54S mutant protein under these conditions showed a binding site size of 34, indicating a binding mode typical of "low-salt" concentrations (Figure 3). At 1 M NaCl, binding of EcoSSB W54S to poly(dT) is weak ($K_{assoc} \approx 5 \times 10^4$ M^{-1}) with a stoichiometry of $n > 60$ as estimated from a simultaneous fit of inverse titrations at 0.37 and 1.35 μM protein assuming a cooperativity parameter ω between 50 and 500. This indicates a shift from the "low-" to "high-salt" binding mode at very high salt concentrations. We conclude that for the "high-salt" binding mode an aromatic residue at position 54 is relevant to overcome the electrostatic repulsion of the dT residues.

Binding of W54F mutant EcoSSB to polynucleotides has been reported earlier by Khamis et al. (1987). They also find a strong binding affinity of W54F for poly(dT), in agreement with our results. While presenting a thorough compilation of the binding constants, the authors do not give the stoichiometries of binding, so a direct comparison is not possible. Optically detected magnetic resonance studies also indicate a stacking interaction between tryptophan-54 and the nucleotide residues (Khamis et al., 1987).

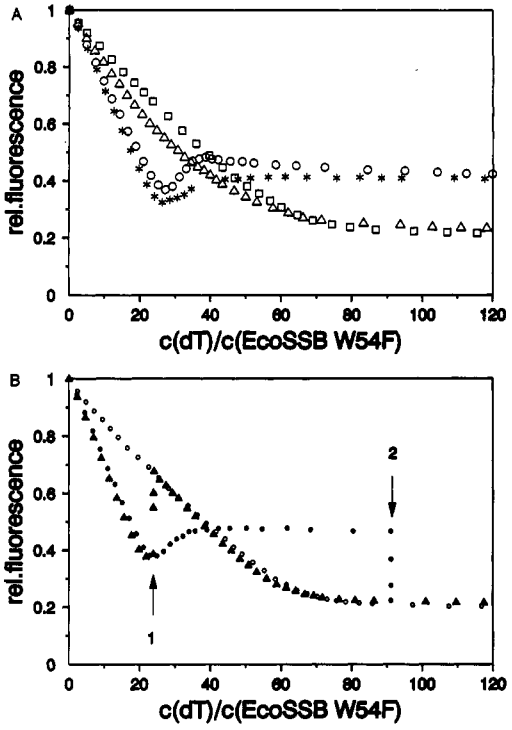

FIGURE 4: Salt dependence of the binding of W54F mutant EcoSSB to poly(dT). (A) Inverse titrations at 0.56 μM protein were carried out at 0.3 M NaCl (□), 0.05 M NaCl (△), 6 mM NaCl with 1.2 mM KP_i (○), and 1 mM NaCl with 1 mM KP_i (*). (B) (▲) An inverse titration at 0.34 μM protein was begun at 1 mM NaCl/1 mM KP_i; after the minimum of fluorescence was reached (1), NaCl and KP_i were added in several steps to a final concentration of 0.3 M NaCl and 0.02 M KP_i; the titration was then continued to saturation. (●) An inverse titration at 0.34 μM protein was carried out at 1 mM NaCl/1 mM KP_i; after saturation was reached (2), NaCl and KP_i were added in several steps to a final concentration of 0.3 M NaCl and 0.02 M KP_i. In both titrations, fluorescence was corrected for dilution when salt was added. (○) Inverse titration at 0.3 M NaCl/0.02 M KP_i for sake of comparison.

It is important to note that for W54S a "low-salt" binding stoichiometry is accompanied by a reduction of fluorescence intensity by 75% (Figures 3 and 5). A similar observation was made for the W54F mutant. While at 0.3 M NaCl this mutant behaves similar to wild-type EcoSSB (Figure 3), below 10 mM NaCl the apparent binding site size is reduced 2-fold and the binding is accompanied by a reduction in fluorescence of 67% (Figure 4). A reduction in fluorescence of over 50% is difficult to perceive with a model in which only two out of four subunits are involved in binding (Lohman et al., 1986a). A similar result was obtained for the Trp40 triple mutant (Figure 10), where a fluorescence reduction of almost 80% is associated with a binding site size of 27.

EcoSSB W40T, W88T, and W135T at "low salt" bind to poly(dT) (data not shown) in a manner very similar to that of wild-type EcoSSB (Figure 2). The titrations with W54F and W54S at "low salt" (Figures 4A and 5), however, reveal a more complex behavior than that expected for a simple interaction having 1 sort of binding mode: addition of poly(dT) to a complex in which 27 nucleotides are covered by 1 mutant EcoSSB protein leads to an increase in fluorescence, and further addition of poly(dT) lets the fluorescence decrease again. When the order of titration was changed, i.e., adding mutant EcoSSB to poly(dT), a similar effect was observed

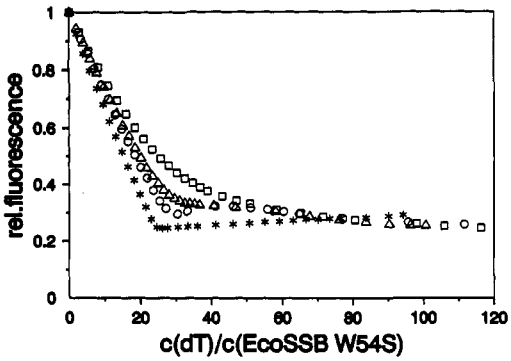

FIGURE 5: Salt dependence of the binding of 0.56 μM W54S mutant EcoSSB to poly(dT). Inverse titrations were performed at 0.3 M NaCl (□), 0.2 M NaCl (△), 0.1 M NaCl (○), and 5 mM NaCl with 0.8 mM KP_i (*).

(Figure 6A). In this case, the increase in fluorescence due to addition of EcoSSB shows a pronounced decline in the region where 30 nucleotides for 1 EcoSSB are available. This observation is not due to a kinetic artifact: at several titration points, we incubated the mixture up to 6000 s (data not shown) and found no slow change in fluorescence. Thus, we exclude that the observed effects are due to insufficient reaction times. Stopped-flow kinetics, in which W54S EcoSSB was presaturated with 30 nucleotides of poly(dT) (Figure 7), showed that the fluorescence enhancement as a consequence of adding more poly(dT) is completed within several seconds.

We tested whether the salt-dependent binding behavior of W54F mutant EcoSSB could be reversed during a titration by addition of salt. In Figure 4B, it is shown that increasing the salt concentration from 1 mM to 0.3 M NaCl completely reconstituted the "high-salt" binding mode at low and high binding densities. In addition, a titration started at 1 mM NaCl could be continued at 0.3 M NaCl with no change compared to a titration started at 0.3 M NaCl.

A minimal model to explain the nonmonotonous titration curves in Figures 4, 5, and 6 contains two binding modes with different binding site sizes and fluorescence properties. Both binding modes have to be cooperative. Figure 8 illustrates the different binding modes.

Figure 6 gives the result of such a fitting procedure for W54F EcoSSB binding to poly(dT) at 3 mM NaCl. At least two "low-salt" binding modes with similar binding constants and small binding site sizes, $n_1 = 27 \pm 2$ (n_{27} mode) and $n_2 = 33 \pm 2$ (n_{33} mode), are required to explain the experimental data. None of these binding modes resembles the "high-salt" binding mode ($n = 65 \pm 4$). The model can be used to simultaneously fit normal and inverse titrations (cf. Materials and Methods) with the same set of n, K, and ω.

It is noteworthy that two binding modes can cause a three-step titration behavior, an initial steep drop of fluorescence followed by an increase and at very high ssDNA/protein ratios again a decrease. This behavior can easily be understood by looking at the concentrations of protein bound in any of the two states (Figure 6). At excess protein, the n_{27} mode is favored despite its lower cooperative affinity ($K_1 \cdot \omega_1$) because it needs less space on the ssDNA matrix. Only a small amount of excess ssDNA is then required to "switch" into the n_{33} mode whose binding affinity to a densely packed polymer ($K_2 \cdot \omega_2$) is higher. At even higher excess of ssDNA, the noncooperatively bound states will be favored, thus shifting the relative ratios of bound protein toward the ratio of intrinsic binding affinities (K_1/K_2). Since the titrations were done under "stoichiometric" conditions, the results of the curve-fitting procedures give reliable estimates only for the relative magnitudes of K_{assoc}

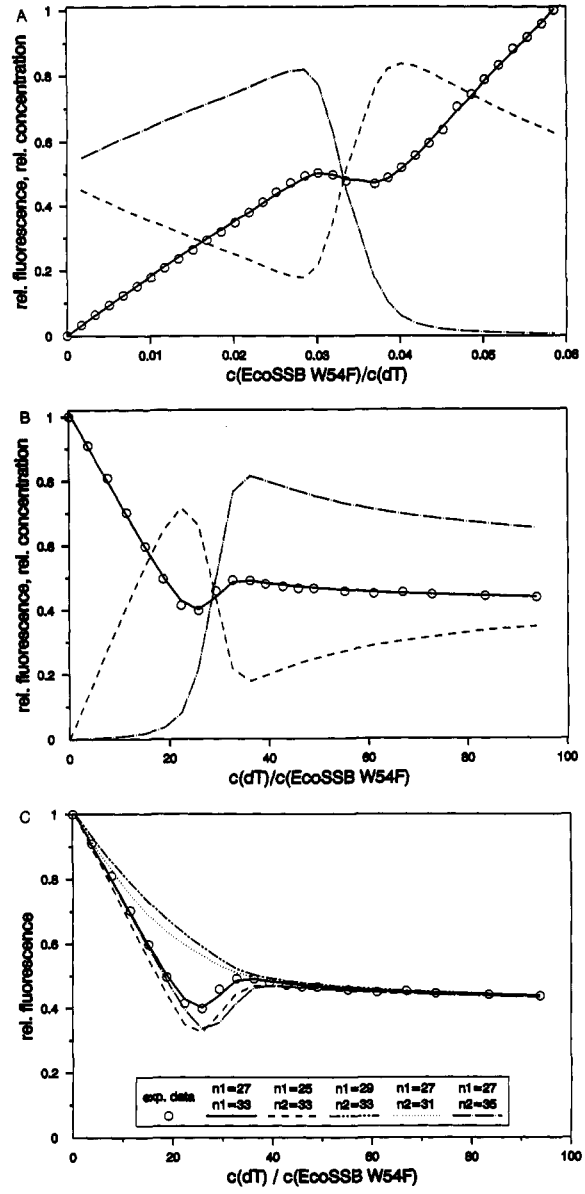

FIGURE 6: Normal (A) and inverse (B) fluorescence titrations of W54F mutant EcoSSB with poly(dT) at 3 mM NaCl with 3 mM KP_i. The solid lines show the best fit using a model where the protein binds in two different binding modes. They were calculated assuming $n_1 = 27$, $K_1 = 1.6 \times 10^7\ M^{-1}$, $\omega_1 = 57$, $f^c_1 = 0.26$, $n_2 = 33$, $K_2 = 1.7 \times 10^7\ M^{-1}$, $\omega_2 = 109$, and $f^c_2 = 0.5$. The dashed and the dash–dotted lines represent the portion of protein bound in the n_{27} mode and the n_{33} mode, respectively. (C) shows simulations of the titration in (B) with n_1 and n_2 being varied by ±2 while keeping the other parameters constant at the values given previously.

and ω necessary to describe the general shape of the curves. However, the binding site sizes can be given within ±2 nucleotides as can be seen from simulations shown in Figure 6C.

The nonmonotonous titration curves at "low salt" could only be observed in those mutant proteins that lacked a tryptophan at position 54 but possessed tryptophan at position 88. Correspondingly, the Trp88 mutant protein, where all three other tryptophan residues are missing, bound poly(dT) at "low salt" in two binding modes with $n_1 = 25 \pm 2$ and $n_2 = 36 \pm 2$ (Figure 9). Of all mutants investigated by us, the observed fluorescence increase upon going from the n_{27} to the n_{33} mode was most pronounced for Trp88. Therefore, we attribute the fluorescence enhancement in this transition to tryptophan at position 88. According to Khamis et al. (1987), tryptophan-88 does not participate in binding of EcoSSB to

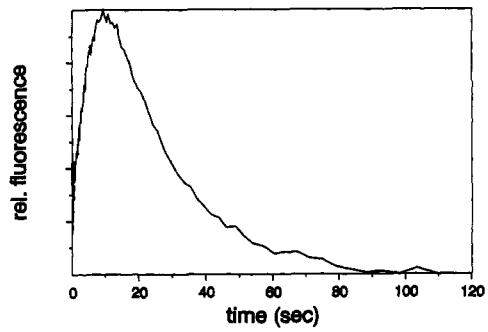

FIGURE 7: Stopped-flow experiment at 0.1 M NaCl, where a complex of 0.5 μM W54S EcoSSB and 15 μM poly(dT) (30 nucleotides per protein tetramer) reacted with a solution containing 58.7 μM poly(dT). This corresponds to a final concentration ratio of 147 nucleotides/EcoSSB.

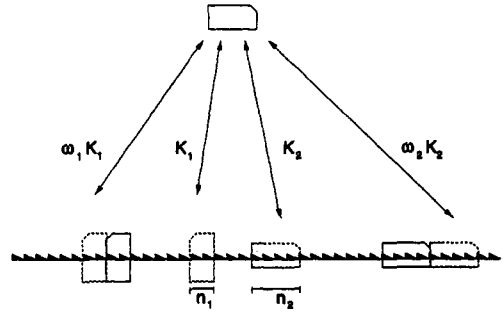

FIGURE 8: Binding of a multidentate ligand to a linear chain molecule in two different binding modes. Each mode is characterized by an intrinsic binding constant (K), a cooperativity parameter (ω), and a binding site size (n). For simplification, cooperativity between proteins bound in different modes was neglected.

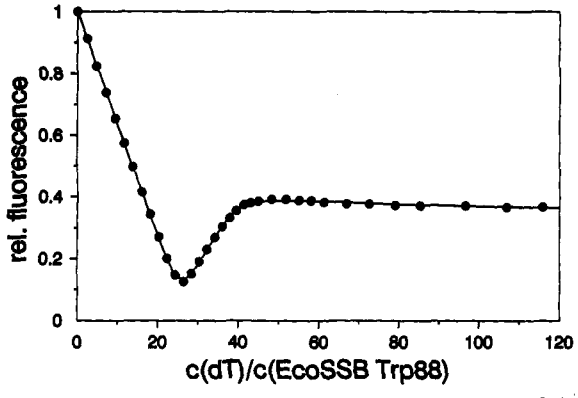

FIGURE 9: Fluorescence titration of the triple mutant Trp88 (0.46 μM) with poly(dT) at 5 mM NaCl and 5 mM KP_i. Nonlinear curve fitting was done using the model in which the protein can bind to poly(dT) in two alternative modes. Parameters used for curve-fitting determined from a simultaneous fit of titrations at 0.12 and 0.46 μM protein were $n_1 = 25$, $K_1 = 3.4 \times 10^7$ M^{-1}, $\omega_1 = 34$, $f^c_1 = 0.07$, $n_2 = 36$, $K_2 = 4.4 \times 10^7$ M^{-1}, $\omega_2 = 66$, and $f^c_2 = 0.53$.

nucleic acids. Our data, in contrast, rather suggest that tryptophan-88 plays an important role in the modulation of binding stoichiometries.

Tryptophan-54 accounts for half of the fluorescence of wild-type EcoSSB. At "low-salt" concentrations, binding of poly(dT) to the Trp54 mutant protein, where all other tryptophan residues have been replaced, reduces the protein fluorescence to 50%. From fluorescence titrations, an apparent binding site size of 38 ± 6 is evaluated (data not shown). A similar titration curve is observed for wild-type EcoSSB. As shown in Figure 2, wild-type EcoSSB at "low-salt" concentrations shows an apparent binding site size of 35 ± 5 nucleotides and a fluorescence quench of 50% at the apparent equivalence

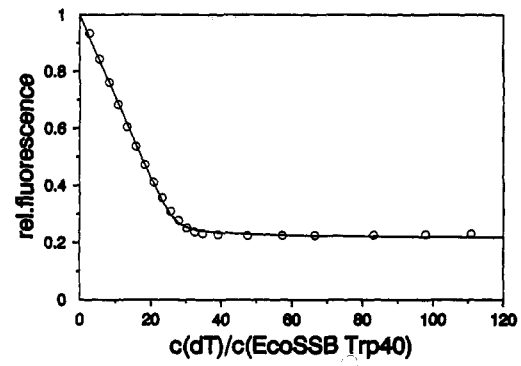

FIGURE 10: Inverse fluorescence titration of the triple mutant EcoSSB Trp40 (0.46 μM) with poly(dT) at 5 mM NaCl and 5 mM KP_i. The solid line represents a fit to three titrations with protein concentrations between 0.76 and 0.23 μM using a binding site size n of 27 and f^c of 0.20.

point. Trp40 mutant EcoSSB, with three out of four tryptophans missing, at "low-salt" concentrations binds to poly(dT) with only one small binding site size ($n = 27$) and a large fluorescence decrease ($f^c = 0.20$) (Figure 10). Our interpretion of these results is that in the presence of tryptophan at position 54 the n_{27} mode is not used or not observable. In the absence of tryptophan-54 and tryptophan-88, the n_{27} mode is used. It is possible that tryptophan-54 interacts with ssDNA in a manner where the n_{33} mode is preferred over the n_{27} mode. Simulations with our two-mode binding model showed that the n_{27} binding mode may be unobservable already when the binding affinity of the n_{33} mode is only larger by a factor of 5.

Closer inspection of the titrations of wild-type protein at "low-salt" concentrations reveals that after reaching the apparent equivalence point the fluorescence of the protein decreases upon further addition of poly(dT). We conclude that at higher ssDNA/protein ratios under these conditions another binding mode with low fluorescence intensity exists. However, due to the large contributions of tryptophan-54 to the fluorescence effects, only monotonous behavior is observed in the titrations. The data can be fitted using two binding modes, the results, however, being ambiguous. In simulations this subtle fluorescence decrease at high ssDNA/protein ratios can equally well be explained by models comprising either an n_{33} mode and a mode with a larger binding site size or an n_{33} mode together with an n_{27} mode. We will have to await further investigations of the binding behavior of wild-type EcoSSB to poly(dT) to clarify this point.

CONCLUSION

Site-directed mutagenesis of *E. coli* single-stranded DNA binding protein demonstrates the coexistence of two binding modes at "low salt". These binding modes have small binding site sizes and comparable affinities. In at least one of these modes, all four subunits of the protein seem to be involved in ssDNA binding. At "high salt", tryptophan at position 54 interacts directly with the ssDNA. This interaction is relevant for the observed large binding site size.

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