Variability in the nucleic acid binding site size and the amount of single-stranded DNA-binding protein in *Escherichia coli*

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The *Escherichia coli* single-stranded DNA binding protein (SSB), essential for DNA replication, recombination and repair, can undergo a thermally induced irreversible conformational change which does not eliminate its biological activity, but changes the number of nucleotides it covers (binding site size) when binding to a single-stranded nucleic acid lattice. The binding site size of native and conformationally changed SSB was also found to be a function of the molecular mass of the polynucleotide, an observation which is unusual for single-stranded DNA binding proteins and will greatly affect the affinity relationship of this protein for nucleic acids. A radioimmunoassay used to quantitate in SSB level in cells revealed the number of SSB tetramers to be larger than initial estimates by a factor of as much as six. All these data suggest that the biological role of SSB and its mechanism of action is by far more complex than originally assumed.

Spin-labeled nucleic acid E. coli *single-stranded DNA binding protein* *Nucleic acid binding site size*

1. INTRODUCTION

The *Escherichia coli* single-stranded DNA binding protein (SSB) is essential for DNA replication, recombination and repair [1–4]. So far two mutations in the *ssb* structural gene are known and both mutations give similar cellular deficiencies [5]. Although the central role of SSB is well acknowledged, little is known about its precise quantity in the cell, nor is there much agreement about the stoichiometry in an SSB–DNA complex. The determination of relevant binding stoichiometries for SSB and nucleic acids is essential for determining the apparent binding constants in such complexes and thereby obtaining the proper affinity relationship of SSB for different nucleic acids. Such relationships are a necessity for proposing detailed mechanisms involving SSB. Here, we establish that the absolute amount of SSB in an *E. coli* cell is considerably larger than originally assumed [6], and that the binding stoichiometry of SSB is by far more complex than generally accepted and does not follow a simple pattern as observed for gene 32 [7] or gene 5 protein [8].

2. MATERIALS AND METHODS

2.1. *Protein purification*

Wild type SSB was prepared from 250 g of the ssb^+ plasmid-containing strain RM140 [9] or from 1 kg of the ssb-1 strain RM121 [9] essentially as described [10] ('boiled protein') or with the following modifications which omitted the boiling step and resulted in unboiled protein (see table 1). Fractions from the blue dextran-Sepharose column containing SSB were pooled and adjusted to 1 M NaCl. A hydroxyapatite column (0.64 $cm^2 \times 3$ cm) was equilibrated with 50 mM imidazole (pH 6.8), 20% glycerol, 0.5 mM dithiothreitol and 1 M NaCl. The sample was loaded and the column washed with starting buffer and then eluted with a 20–150 mM potassium phosphate (pH 6.5) gradient containing 20% glycerol, 0.5 mM

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dithiothreitol and 0.2 M NaCl. The SSB elutes from this column at 50–60 mM as a homogeneous protein.

2.2. *Radioimmunoassay*

Antibody to SSB was produced in rabbits, and immunoglobulin G (IgG) was purified. Goat anti-rabbit gamma globulin (GARGG) was purchased from Calbiochem. A double antibody radioimmunoassay (RIA) was used to measure directly the amount of SSB present in crude lysates. Reactions (0.5 ml) were carried out at 4°C in 1.5 ml Eppendorf tubes. To the appropriate amount of buffer [20 mM sodium phosphate (pH 7.4), NaCl (either 0 or 2 M as indicated), 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% gelatin, 1% Triton X-100)], 10–25 ng of ^{125}I-SSB (50000 cpm) was added. To this either known amounts of purified SSB or aliquots of crude extract were added, mixed and incubated 60 min at 4°C. SSB-specific IgG (approx. 0.5 μg) was added such that 50–70% of the radiolabeled SSB was precipitated in the control tubes. The primary binding reaction was allowed to occur overnight. The next day IgG (3.5 μg) purified from pre-immunized bleeds was added as carrier, and GARGG was added to precipitate the rabbit IgG in an overnight incubation. The tubes were centrifuged, the pellet washed twice with buffer and counted. Extract samples were between 50 and 300 μl. Five different volumes of extract were measured in duplicate, and the quantity of SSB in these extracts calculated from the values which resulted in 20–70% inhibition of the precipitation of the radiolabeled SSB.

2.3. *ESR measurements*

All ESR spectra were obtained with a Varian E-104 Century Series spectrometer which was interfaced with an Apple II plus microcomputer [11]. The titrations were performed in an E-258-3 Varian flat quartz cell using an E-238 cavity. The SSB ligand was introduced in incremental (2–5 μl) additions to a known molar quantity of (1 dT,dT)$_n$ or (DUAP,dT)$_n$ (10–20 nmol nucleotides) using as starting volume 170 μl. Mixing of the components was achieved with a plastic tuberculin syringe temporarily attached to the neck of the ESR cell. Solutions were gently mixed for approximately 2 min. To assure equilibrium between the components in solutions several time-course experiments were performed which involved several different mixing times. These tests showed that a 2-min equilibration time is entirely sufficient. The ESR spectra were analyzed in terms of a two-state model with a published algorithm [12].

2.4. *Absorbance measurements*

UV absorbance was monitored on a Gilford 250 spectrophotometer. The UV titrations were done by adding aliquots of SSB (2–5 μl) to 200 μl of a polynucleotide solution, and mixing gently either with a syringe or by inverting the cuvette several times. Both mixing procedures gave the same results.

2.5. *Concentration determinations*

Nucleic acid concentrations were calculated from solution absorbancies with the following extinction coefficients ($\times 10^{-3}$ M$^{-1}\cdot$cm^{-1}): ϵ_{265} = 8.7 for (dT)$_n$, (1dT,dT)$_n$ and (DUAP,dT)$_n$; ϵ_{259} = 7.4 for fd DNA. SSB protein concentrations were determined by the Lowry method yielding an extinction coefficient ϵ_{280} = 28500 per monomer, which is in good agreement with the ϵ published by Ruyechan and Wetmur [13] after including in the calculation the M_r value of 18873 for the monomer [14], but is considerably larger than the ϵ value reported by Krauss et al. [15]. Recently, an ϵ_{280} = 27024 was reported for either SSB or SSB-1 based on the known tyrosine and tryptophan content of SSB [5], and a year earlier the same laboratory reported an ϵ_{280} = 30250 for SSB [16].

2.6. *Nucleic acids and their molecular mass determination*

(dT)$_n$ was purchased from P-L Biochemicals and fd DNA from Miles Laboratories. (1dT,dT)$_n$ was prepared by chemical modification of (dT)$_n$ [17]. (DUAP,dT)$_n$ was obtained through enzymatic synthesis using the procedure published for (DUTT,dT)$_n$ [18]. The synthesis of DUAP, an analog of DUTT [19], will be published elsewhere. The average M_r of the polynucleotides was determined by gel electrophoresis. Both (dT)$_n$ and (1dT,dT)$_n$ have M_r values of 150000–200000, whereas (DUAP,dT)$_n$ has an M_r of 50000–100000. The amount of spin incorporation is indicated in the figure legends.

3. RESULTS AND DISCUSSION

In fig.1 the fraction F of spin-labeled polynucleotides complexed with SSB or SSB-1 is shown for ESR titrations with $(1dT,dT)_n$ and (DUAP,dT). The saturation of the spin-labeled nucleic acid lattice follows a two-state model as observed earlier for gene 32 and gene 5 protein [7,8]. It is obvious that the two lattices are saturated at two significantly different P/N ratios. For the wild type SSB the dependence of F on P/N is essentially linear until about $F = 0.8$ when a slight deviation from linearity becomes noticeable. Such departure from linearity was already observed earlier for gene 32 and gene 5 protein and is ascribed to end group effects. A linear extrapolation of the data gives a P/N ratio of 0.02 and 0.04 for $(DUAP,dT)_n$ and $(1dT,dT)_n$, respectively, at $F = 1$. This corresponds to a binding site size of 25 ± 10% for $(DUAP,dT)_n$ and 50 ± 10% for $(1dT,dT)_n$.

To evaluate potential perturbation of the probe on the nucleic acid matrix with respect to binding stoichiometry, UV absorbance titrations were performed with $(DUAP,dT)_n$, $(dT)_n$, $(1dT,dT)_n$, and fd DNA. Two characteristic titration results are shown with fig.2, and the data are summarized in table 1. The results indicate that the same binding stoichiometry is observed with $(dT)_n$ and $(1dT,dT)_n$ suggesting that the label has no effect on the binding site size at these low levels of spin incorporation. A similar conclusion was made earlier for gene 32 protein [7].

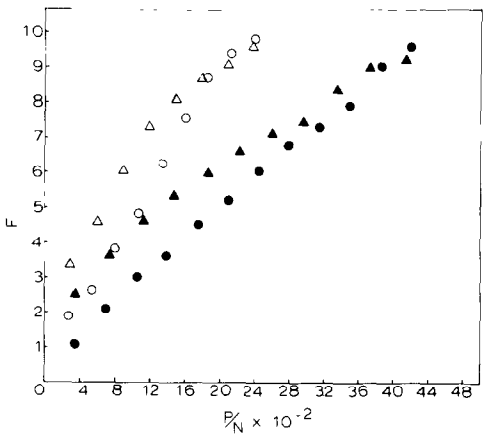

Fig.1. Plot of F, fraction of complexed spin labeled polynucleotide, vs SSB tetramer/nucleotide bases based on ESR titration data. $(1dT,dT)_n$ (1dT/dT = 0.025 ± 0.003; M_r 150000–200000) with wild type SSB (○—○) and with SSB-1 (△—△); $(DUAPT,dT)_n$ (DUAPT/dT = 0.03 ± 0.003; M_r 50000–100000) with wild type SSB (●—●) and with SSB-1 (▲—▲). The titrations were done at 23°C in 125 mM NaCl, 20 mM Tris-HCl (pH 8.1), 1 mM EDTA, 0.1 mM DTT, 10% (w/v) glycerol, 0.05% (w/v) Triton.

Two striking features are apparent from table 1, which summarizes the binding site size data. First, the binding site size depends on the molecular mass of the nucleic lattice, the largest site size being observed with fd DNA with its molecular mass of 2.1×10^6 Da. Second, SSB which was not boiled during the isolation process gives a systematically lower site size with all lattices tested than SSB which was subjected to a boiling step. Boiling of SSB, which was isolated without a boiling step causes the same increased site size effect as SSB (boiled) even after letting the SSB solution re-equilibrate for several weeks at 4°C. This observation suggests that SSB will undergo a thermally induced irreversible conformational change without, however, eliminating its biological activity [10]. Also, the variation of the binding site size from 25 to 100 per tetramer considerably complicates the accurate calculation of binding constants, which is important for a proper understanding of the mechanism of action of this protein.

A radioimmunoassay was developed to quantitate the levels of SSB in the cell. In exponentially growing *E. coli* we obtained values ranging from 0.22 to 0.49 ng of SSB per μg of soluble protein of cells grown in minimal M9 medium, depending on

Table 1

Binding site size of wild type SSB[a]

	SSB	
	Unboiled[b]	Boiled[b]
$(DUAP,dT)_n$	25 ± 10%	45 ± 10%
$(dT)_n$	50 ± 10%	70 ± 10%
$(1dT,dT)_n$	50 ± 10%	–
fd DNA	75 ± 10%	100 ± 10%

[a] In 125 mM NaCl (or 200 mM NaCl), 20 mM Tris–HCl (pH 8.1), 1 mM EDTA, 0.1 mM DTT

[b] For preparation see section 2

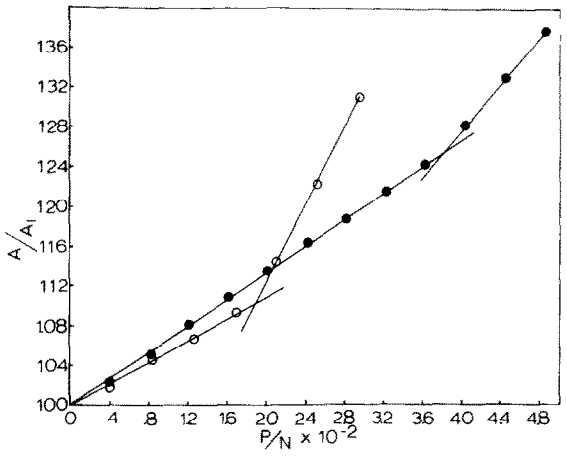

Fig.2. UV absorbance titrations of $(1dT,dT)_n$ (1dT/dT = 0.025 ± 0.003; M_r 150000–200000) (○—○) and $(DUAPT,dT)_n$ (DUAPT/dT = 0.03 ± 0.003; M_r 50000–100000) (●—●) with wild type SSB. The absorbance ratio of final vs initial absorbance at 260 nm is plotted as a function of SSB tetramer/nucleotide bases. The titrations were done at 23°C in 125 mM NaCl, 20 mM Tris–HCl (pH 8.1), 1 mM EDTA, 0.1 mM DTT.

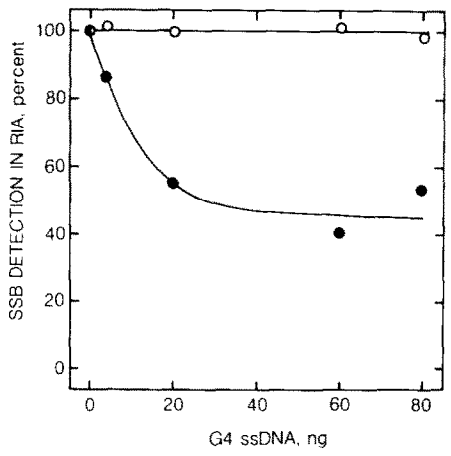

Fig.3. Increasing concentrations of G4 ssDNA were incubated with unlabeled SSB (20 ng) at 4°C for 60 min in the presence or absence of salt followed by RIA in the presence (○—○) or absence (●—●) of 2 M NaCl.

which strain was examined (table 2). When these strains were grown in rich medium (Luria broth), values of 0.81–0.95 ng/μg were found. By counting cells directly in a Petroff-Hauser counting chamber and using an M_r = 18873 for SSB monomers [14], the number of such tetramers per cell was determined. We estimate the number of SSB tetramers per cell in a log phase culture to be at a minimum of 1000, and possibly twice this value when grown in rich media. These values are larger than the initial estimates [6] by a factor of as much as six. Because SSB binds tightly to single-stranded DNA, the possibility that SSB would not be efficiently detected in the presence of DNA was considered. To test this, unlabeled SSB was preincubated with increasing concentrations of G4 DNA. Then the detection of SSB by radioimmunoassay was examined in the presence or absence of high salt (2 M) (fig.3). Using 20 ng SSB, only 50% of the SSB present could be detected at DNA concentrations greater than

Table 2

Cell strain	Media	Total soluble protein (μg/μl)	SSB in extract[a] (ng/μl)	SSB/total protein (ng/μg)
C600	M9	0.24 ± 0.006	0.12 ± 0.01	0.49 ± 0.039
C600/pSR6[b]	M9	0.13 ± 0.006	1.1 ± 0.17	8.6 ± 1.2
C600	LB	0.31 ± 0.047	0.28 ± 0.032	0.86 ± 0.14
C600/pSR6	LB	0.20 ± 0.008	6.1 ± 0.51	31 ± 2.7
W3110	M9	0.45 ± 0.036	0.10 ± 0.009	0.22 ± 0.025
AB1157	M9	0.41 ± 0.011	0.13 ± 0.025	0.31 ± 0.055

[a] SSB was determined by RIA in 2 M NaCl using cleared high salt lysates prepared from sonicated cells

[b] ssb plasmid [9]

40 ng/ml. These results offer an explanation of the underestimation of the SSB levels.

These data, along with those of binding stoichiometries, indicate that there is sufficient SSB in an *E. coli* cell grown in rich media to bind as much as 150000 nucleotides of its single-stranded DNA. After taking into consideration the number of replication forks and the size of their single-stranded regions, it becomes apparent that there is considerably more SSB in a cell than required for replication. This large excess of SSB may be needed in processes such as recombination and repair. The dependence of the binding site size of SSB on the overall size of the nucleic acid lattice also suggests that the biological role of SSB and its mechanism of action is far more complex than originally assumed.

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REFERENCES

[1] Meyer, R.R., Glassberg, J. and Kornberg, A. (1979) Proc. Natl. Acad. Sci. USA 76, 1702–1705.
[2] Glassberg, J., Meyer, R.R. and Kornberg, A. (1979) J. Bacteriol. 140, 14–19.
[3] Golup, E.I. and Low, K.B. (1983) Proc. Natl. Acad. Sci. USA 80, 1401–1405.
[4] Whittier, R.F. and Chase, J.W. (1983) Mol. Gen. Genet. 190, 101–111.
[5] Williams, K.R., Murphy, J.B. and Chase, J.W. (1984) J. Biol. Chem. 259, 11804–11811.
[6] Weiner, J.H., Bertsch, L.L. and Kornberg, A. (1975) J. Biol. Chem. 250, 1972–1980.
[7] Bobst, A.M., Langemeier, P.W., Warwick-Koochaki, P.E., Bobst, E.V. and Ireland, J.C. (1982) J. Biol. Chem. 257, 6184–6193.
[8] Bobst, A.M., Ireland, J.C. and Bobst, E.V. (1984) J. Biol. Chem. 259, 2130–2134.
[9] Meyer, R.R., Voegele, D.W., Ruben, S.M., Rein, D.C. and Trela, J.M. (1982) Mutat. Res. 94, 299–313.
[10] Meyer, R.R., Glassberg, J., Scott, J.V. and Kornberg, A. (1980) J. Biol. Chem. 255, 2897–2901.
[11] Ireland, J.C., Willett, J.A. and Bobst, A.M. (1983) J. Biochem. Biophys. Methods 8, 49–56.
[12] Bobst, A.M., Sinha, T.K., Langemeier, P.W. and Prairie, R.L. (1980) Comput. Chem. 4, 45–50.
[13] Ruyechan, W.T. and Wetmur, J.G. (1975) Biochemistry 25, 5529–5534.
[14] Sancar, A., Williams, K.R., Chase, J.W. and Rupp, W.D. (1981) Proc. Natl. Acad. Sci. USA 78, 4274–4278.
[15] Krauss, G., Sindermann, H., Schomburg, U. and Maass, G. (1981) Biochemistry 20, 5346–5352.
[16] Williams, K.R., Spicer, E.K., LoPresti, M.B., Guggenheimer, R.A. and Chase, J.W. (1983) J. Biol. Chem. 258, 3346–3355.
[17] Bobst, A.M. (1979) in: Spin Labeling II: Theory and Applications (Berliner, L.J. ed.) pp.291–345, Academic Press, New York.
[18] Bobst, A.M., Kao, S.-C., Toppin, R.C., Ireland, J.C. and Thomas, I.E. (1984) J. Mol. Biol. 173, 63–74.
[19] Toppin, C.R., Thomas, I.E., Bobst, E.V. and Bobst, A.M. (1983) Int. J. Biol. Macromol. 5, 33–36.