Functional Domains of *Escherichia coli* Single-Stranded DNA Binding Protein As Assessed by Analyses of the Deletion Mutants†

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ABSTRACT: A series of C- and N-terminal deletion mutants of *Escherichia coli* single-stranded DNA binding protein (SSB) was constructed, purified, and characterized in terms of ability to self-multimerize and to bind to DNA. High-performance gel filtration chromatography revealed that the amino acids 89−105 play a key role in the maintenance of homotetramer for native SSB of 177 amino acids. Interestingly, all of the N-terminal deletion mutants studied here were eluted as octamers, indicating that the N-terminal 11 residues are involved in the prevention of the formation of octamers. The binding of SSB and its deletion mutant proteins to single-stranded $d(T)_n$ was examined by gel mobility shift assay and circular dichroism spectroscopy. C-terminal deletion mutant proteins, SSB1−135 and SSB1−115, maintained high affinity and may be wrapped by single-stranded DNA (ssDNA) in the same way as in the case of native SSB. In contrast, deletion of the C-terminal region (residues 89−115) or N-terminal region (residues 1−11) caused a dramatic decrease in the binding affinity. Furthermore, two different stoichiometries of SSB in the complexes with $d(T)_{64}$, but not with $d(T)_{32}$, were observed for native SSB, SSB1−135, SSB1−115, and SSB37−177, suggesting that the $(SSB)_{65}$ and $(SSB)_{35}$ binding modes, as previously demonstrated [Lohman, T. M., & Overman, L. B. (1985) *J. Biol. Chem.* 260, 3594−3603; Bujalowski, W., & Lohman, T. M. (1986) *Biochemistry* 25, 7799−7802], occurred at lower and higher SSB concentrations, respectively. A functional map for SSB molecule was presented and discussed.

Proteins that specifically bind to single-stranded DNA (ssDNA)[1] play an essential role in DNA replication, repair, and recombination of both prokaryotes and eukaryotes. The single-stranded DNA binding protein of *Escherichia coli* (SSB) is one of the most intensively studied among these proteins [see reviews by Meyer and Laine (1990), Lohman and Bujalowski (1990), and Lohman and Ferrari (1994)]. In physiological conditions SSB exists as a homotetramer of a subunit of M_r 18 843 (177 amino acid residues) and binds specifically and cooperatively to ssDNA.

Lohman and his colleagues [see reviews by Lohman and Bujalowski (1990) and Lohman and Ferrari (1994)] showed the variety of the interactions of SSB with ssDNA. For example, increasing salt concentration reduces the cooperativity of SSB binding to ssDNA (Bujalowski & Lohman, 1989a,b). The binding stoichiometry is also affected by salt concentration, type of cation and anion, pH and binding density of SSB (Bujalowski & Lohman, 1986; Bujalowski et al., 1988; Curth et al., 1993; Lohman & Bujalowski, 1994). Three distinct binding modes with site sizes of approximately 35, 56, and 65 nucleotides per tetramer are identified under various conditions [Lohman & Overman, 1985; Bujalowski & Lohman, 1986; Bujalowski et al., 1988; also see review by Lohman and Bujalowski (1990)]. It was suggested that the switching among these binding modes might be involved in biological processes (Lohman & Bujalowski, 1990; Ferrari et al., 1994).

Notably, SSB activates N4 early promoters by stabilizing a DNA hairpin required for promoter recognition by viron RNA polymerase, and no other single-stranded DNA binding proteins can substitute it (Markiewicz et al., 1992; Alexandra et al., 1996). Moreover, SSB alters the structure of intramolecular DNA triplexes in supercoiled plasmids (Klysik & Shimizu, 1993). Thus, the interaction of SSB with DNA is not simple and is not yet been well understood.

A proteolytic study has demonstrated that the N-terminal peptides, SSB_T and SSB_C, obtained from digestions by trypsin at Arg-115 and by chymotrypsin at Trp-135, respectively, still preserve ssDNA binding ability, indicating that the ssDNA binding domain is located in the N-terminal 115 amino acids (Williams et al., 1983). Studies by site-directed mutagenesis of each tryptophan residue to phenylalanine have shown that Trp-40 and -54 are essential for high-affinity binding to ssDNA, whereas Trp-88 and -135 are not (Casas-Finet et al., 1987; Khamis et al., 1987). Furthermore, the substitution of alanine for Phe-60 or of leucine for His-55 results in a decrease in binding affinity by 3 orders of magnitude (Casas-Finet et al., 1987). Moreover, the *ssb-1* mutation (His-55 to tyrosine) decreases the stability of the

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[1] Abbreviations: SSB, *E. coli* single-stranded DNA binding protein, ssDNA, single-stranded DNA; PCR, polymerase chain reaction. SD sequence, Shine−Dalgarno sequence; M_r, molecular weight; IPTG, isopropyl 1-thio-β-D-galactopyranoside; SDS−PAGE, SDS−polyacrylamide gel electrophoresis.

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SSB tetramer (Williams et al., 1984; Bujalowski & Lohman, 1991a), and the resultant SSB-1 monomer still binds to ssDNA (Bujalowski & Lohman, 1991b). These analyses of point mutants give important but limited information on the binding regions in the protein. Thus, a global view of the structure of SSB such as functional domains remains to be clarified.

Herein, we constructed and purified a series of N- and C-terminal deletion mutants of SSB using T7 overexpression system. Their affinity for single-stranded oligo $d(T)_n$ was characterized by gel mobility shift assay and by circular dichroism spectroscopy. Their oligomerization was monitored by high-performance gel filtration chromatography. On the basis of these results, a functional domain map for SSB molecule was presented and discussed.

MATERIALS AND METHODS

Construction of Plasmids for Expression of Native and Deletion Mutant SSB Proteins

All DNA fragments containing the *ssb* coding sequence were prepared by polymerase chain reaction (PCR) using plasmid pNI6 (Shimamoto et al., 1987) that carries the entire *ssb* gene of *E. coli* (Sancar et al., 1981) as a template.

The PCR products for native SSB and the C-terminal deletion mutants (SSB1−135, SSB1−115, SSB1−105, and SSB1−88) were digested with *Xho*I and *Hin*dIII or *Sal*I and were ligated into pT7-5 (Tabor & Richardson, 1985; Tabor, 1989) between *Xho*I and *Hin*dIII sites or between *Xho*I and *Sal*I sites. These constructs have the T7 promoter with the *ssb* translation initiation region.

The PCR products for the N-terminal deletion mutants (SSB12−177, SSB37−177, SSB37−115, and SSB37−105) were digested with *Nde*I and *Hin*dIII or *Sal*I and were ligated into pT7-7 (Tabor & Richardson, 1985; Tabor, 1989) between *Nde*I and *Hin*dIII or between *Nde*I and *Sal*I. These constructs have the T7 promoter with the T7 translation initiation region.

All of the plasmids constructed here were checked by DNA sequencing (Sambrook et al. 1990). The *ssb* coding sequence for all of the deletion mutant proteins was verified to be identical to that for wild type SSB, except that SSB37−115 had a point mutation of Ser (ACC) to Pro (CCC) at amino acid residue 108.

Expression and Purification of Native and Deletion Mutant SSB Proteins

E. coli strain BL21 (DE3) pLysS was used for expression of the native SSB and its deletion mutants. The transformed cells were incubated at 37 °C in 2 L of LB medium containing 100 μg/mL of ampicillin. When the culture reached at $OD_{600} = 0.5$, expression was induced by adding 1 mM IPTG. After further incubation for 3 h, cells were harvested and stored at −80 °C until use. All SSB mutant proteins were purified as described previously (Shimamoto et al., 1987) with the following modifications.

Native SSB, SSB1−135, SSB1−115, SSB12−177, SSB37−177, and SSB37−115 were precipitated from the supernatant of the cell lysate (60 mL) by incubation with 0.27 g/mL of ammonium sulfate for 30 min and centrifugation at 18 000*g* for 30 min. The pellets were washed subsequently with 2.5 mL of 20 w/w %, 1.25 mL of 20 w/w %, and 1 mL of 16 w/w % ammonium sulfate in a T-buffer [50 mM Tris-HCl (pH 7.5), 10 v/v % glycerol, 1 mM EDTA and 10 mM 2-mercaptoethanol]. After dialysis against I-buffer [50 mM imidazole (pH 6.9), 20 v/v % glycerol, 1 mM EDTA, and 10 mM 2-mercaptoethanol] containing 200 mM NaCl, the native SSB, SSB1−135, SSB1−115, and SSB37−115 were subjected to phosphocellulose (Whatman) column (2.5 cm × 25 cm) chromatography and eluted with a linear gradient of 0.05−0.6 M NaCl in the same buffer (300 mL each). SSB12−177 and SSB37−177 were subjected to a DEAE-Cellulofine (Seikagakukogyo, Japan) column (2.5 cm × 25 cm) chromatography and eluted with a linear gradient of 0.05−0.6 M NaCl in the same buffer (300 mL each).

SSB1−105, SSB1−88, and SSB37−105 overexpressed in inclusion bodies were recovered in pellet from the cell lysate (60 mL) by centrifugation at 18 000*g*, and washed twice with 30 mL of H-buffer (50 mM Na_2HPO_4, 20 v/v % glycerol, and 400 mM NaCl) and once with 30 mL of L-buffer (50 mM NaH_2PO_4, 20 v/v % glycerol, and 400 mM NaCl). The precipitates for SSB1−105 and SSB1−88 were solubilized with 15 mL of I-buffer containing 3 M guanidine hydrochloride, whereas SSB37−105 was solubilized with 15 mL of I-buffer containing 6 M urea. The solutions were then centrifuged at 18 000*g* for 20 min at 4 °C, and the supernatant was diluted 10-fold with I-buffer. SSB1−105 and SSB1−88 were then subjected to phosphocellulose column (2.5 cm × 25 cm) chromatography and eluted with a linear gradient of 0.05−1.0 M NaCl in I-buffer (300 mL each). The protein SSB37−105 was subjected to DEAE-Cellulofine column (2.5 cm × 25 cm) chromatography and eluted with a linear gradient of 0.05−0.6 M NaCl in the same buffer (300 mL each).

The peak fractions containing native and deletion mutant SSB proteins were further subjected to Heparin-Sepharose CL-6B (Pharmacia) column (1.0 cm × 20 cm) chromatography and eluted with a linear gradient of 0.05−1.0 M NaCl in I-buffer (120 mL each).

The purity of the proteins was checked by SDS−polyacrylamide gel electrophoresis (PAGE) (Figure 1). Each SSB mutant protein was also checked by the immunoblotting using anti-SSB serum (Shimamoto et al., 1987). Protein concentration of native SSB was determined by UV absorbance using an extinction coefficient of 29 200 M^{-1} cm^{-1} at 280 nm (Shimamoto et al., 1987). Concentrations of the SSB deletion mutants were determined by the BAC method (Pierce) using native SSB as a standard.

CD spectra of native SSB and mutant proteins were measured as a function of guanidine hydrochloride concentration to conform refolding of proteins expressed in inclusion bodies.

Gel Filtration Chromatography

Purified native SSB and its deletion mutants at 5 μM protein concentration were applied on a Superdex 75 gel filtration column using a Smart system (Pharmacia). Proteins were eluted with a high salt buffer containing 300 mM NaCl, 0.1 mM EDTA, and 10 mM Tris-HCl (pH 7.5) to avoid a nonspecific interaction of proteins with the gel matrix. Fractions of 0.05 mL were collected at a flow rate of 0.05 mL/min, and applied on SDS−polyacrylamide gel electrophoresis (SDS−PAGE). Phosphorylase B, conalbumin,

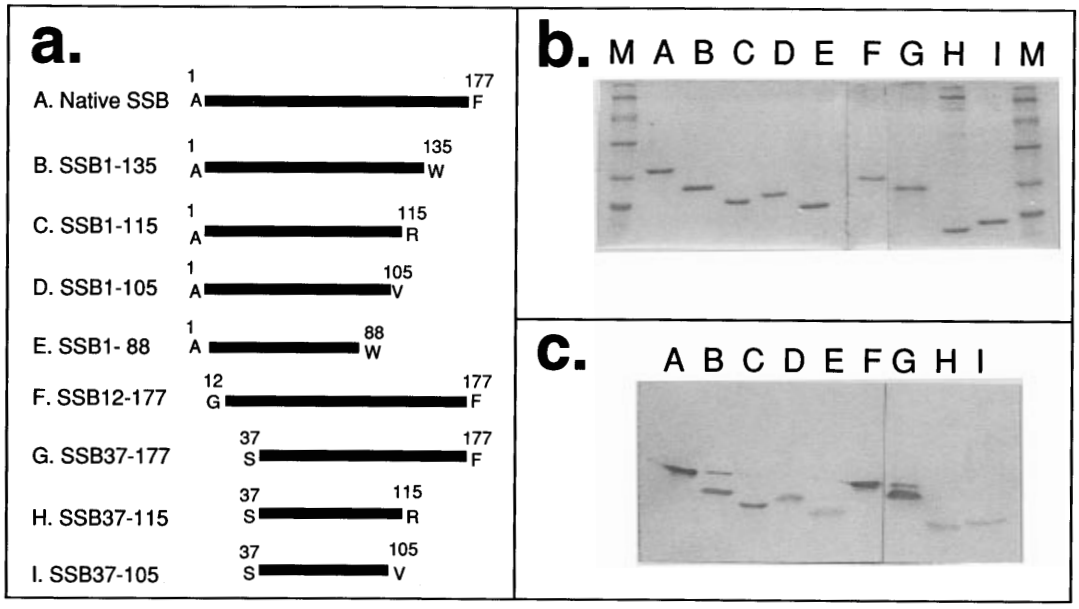

FIGURE 1: Native SSB and its deletion derivatives used in this study. (a) Abbreviations for deletion mutants of SSB as indicated, (b) Coomassie Brilliant Blue stain of SDS−polyacrylamide gel electrophoresis, and (c) immunoblot analysis. Amino acid sequences of all the mutant proteins were identical to wild type SSB, except that SSB37−115 had a Ser to Pro mutation at amino acid residue 108. Molecular weight markers used in panel (b) are phosphorylase B (M_r 94 000), albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), trypsin inhibitor (M_r 20 100), and α-lactglobin (M_r 14 400).

ovalbumin, and cytochrome *C* were used as molecular weight markers.

Gel Mobility Shift Assay

Oligonucleotides were radioactively labeled at the 5′ terminus with T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-^{32}P]ATP (4500 Ci/mmol, ICN Pharmaceuticals, Inc.), and unreacted ATP was removed by passage through a Sephadex G-50 (Pharmacia) spun column (Sambrook et al., 1990). The oligonucleotides (0.5 pmol each in total volume of 50 μL, *i.e.*, 1.0×10^{-8} M) were mixed in a binding buffer [50 mM NaCl, 0.1 mM EDTA, and 10 mM Tris-HCl (pH 8.0)]. Then, the DNA mixture was titrated with SSB. After incubation on ice for 30 min, each mixture was loaded and run on an 12% polyacrylamide gel electrophoresis at 4 °C. The DNA bands were visualized by autoradiography.

CD Measurements

CD spectra were recorded at 22 °C on a JASCO J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd). $d(T)_{32}$ (final concentration, 1 μM) was mixed in a binding buffer [50 mM NaCl, 0.1 mM EDTA, and 10 mM Tris-HCl (pH 8.0)]. Then, the DNA solution was titrated with concentrated solution of the proteins. The CD difference spectra of the proteins−ssDNA complex were recorded as the mean residue ellipticity of the ssDNA.

RESULTS

Expression and Purification of Native and Deletion Mutant SSB Proteins

Three series of SSB deletion mutants, *i.e.*, C-terminal, N-terminal, and both C- and N-terminal deletions are shown in Figure 1a. Native SSB and C-terminal deletion mutants were expressed using T7 promoter with the *ssb* translation initiation region, while N-terminal deletion mutants were expressed using the T7 promoter with the T7 translation initiation region. The N-terminal deletion mutants were expressed at levels 2−5-fold higher than native SSB and C-terminal deletion mutants. The difference in SD sequences may account for the difference in the degree of expression of proteins.

The purity of the proteins was checked by SDS−PAGE and immunoblot as shown in Figure 1b and c. Deletion mutants SSB1−105 and SSB37−105 migrated more slowly than expected from their molecular weights by unknown reason.

Analysis of Multimerization by High-Performance Gel Filtration Chromatography

To characterize the regions required for tetramerization of SSB, the series of deletion mutants were analyzed by high-performance gel filtration chromatography to estimate apparent molecular weights. Multimer formation was determined by the ratio of estimated M_r to monomer M_r as shown in Table 1. Native SSB was eluted normally at a volume corresponding to tetramer, M_r = 78 000. The C-terminal deletion mutants, SSB1−135 and SSB1−115, were eluted at volumes corresponding to trimers, M_r = 46 000 and 38 000, respectively. These behaviors in gel filtration are in good agreement with the previous report that N-terminal fragments, SSB_C (1−135 amino acid residues) and SSB_T (1−115 amino acid residues), obtained from digestions of SSB with chymotrypsin and trypsin, respectively, were eluted in the position of trimeric form (Williams et al., 1983).

The trimer can be interpreted in three ways, the true trimer, a tetramer with an compacted conformation or a mixture of tetramer and dimer rapidly equilibrated. Neither the true trimer with a 3-fold symmetry nor the 2+1 type trimer may be harmonized with the tetrameric structure of the native SSB molecule. Since SSB1−105 maintained tetrameric form as eluted at volume corresponding to M_r = 44 500, the

Table 1: Multimer Formation and Relative Binding Affinty of *E. coli* SSB and Its Mutant Proteins

SSB mutants	M_r estimated[a]	M_r monomer	multimer formation[b]	relative binding affinity[d]
native SSB	78 000	18 843	4.1 (tetramer)	1
SSB1−135	46 000	14 463	3.2 (tetramer)[c]	1/2
SSB1−115	38 000	12 759	3.0 (tetramer)[c]	1/2
SSB1−105	44 500	11 770	3.8 (tetramer)	1/100
SSB1−88	10 500	9 877	1.1 (monomer)	<1/500
SSB12−177	140 000	17 706	7.9 (octamer)	1/200
SSB37−177	130 000	16 796	7.7 (octamer)	1/50
SSB37−115	72 000	9 081	7.9 (octamer)	1/200
SSB37−105	87 000	8 351	10.4 (octamer)	not detectable
	33 000		4.0 (tetramer)	

[a] The molecular weight (M_r) was estimated from the elution profile of Superdex 75 gel filtration chromatography. [b] Multimer formation was determined by the ratio of estimated M_r to monomer M_r. [c] SSB1−135 and SSB1−115 would exist as tetramers with compacted conformations or as a rapidly equilibrating tetramer/dimer mixture, and hence they were regarded as putative tetramers (see text). [d] Relative binding affinity of SSB and its deletion mutants for a single-stranded $d(T)_{32}$ was determined by gel retardation assay (see text).

compacted tetramers of SSB1−135 and SSB1−115 would be plausible. This idea is also consistent with a recent report by Curth et al. (1996) that chemical cross-linking tetramers of EcoSSB Q152* (1−151 residues) and EcoSSB G117* (1−116 residues) were observed. Thus, as shown in Table 1, we regarded SSB1−115 and SSB1−135 as putative tetramers. SSB1−88 was eluted at volumes corresponding to monomer (Table 1). These results indicate that the deletion of residues 89−105 causes instability of the tetrameric structure of the SSB.

On the other hand, the N-terminal deletion mutants, SSB12−177, SSB37−177, and SSB37−115 were eluted as octamers (Table 1), indicating that the deletion of the N-terminal 11 amino acids seemed to dimerize tetramers. Interestingly, SSB37−105 was eluted as two peaks at molecular weights corresponding to tetramer and octamer. The relative intensity of the two peaks varied in different protein concentrations at injection, *i.e.*, tetramer increased at lower concentration but decreased at higher concentration, leading to a slow equilibrium between tetramer and octamer.

Binding Affinity and Modes of SSB and Its Deletion Mutants to Oligonucleotide $d(T)_n$

To identify the DNA binding domain of SSB, we measured the binding affinities of SSB and its deletion mutants to $d(T)_{32}$ by gel mobility shift assay (Figure 2). The band of native SSB−$d(T)_{32}$ complex was retarded by the stoichiometric amounts of SSB, and the free $d(T)_{32}$ completely disappeared at [SSB]/[ssDNA] = 4. The C-terminal deletion mutants, SSB1−135 and SSB1−115, completely retarded the DNA at [SSB]/[ssDNA] = 4−8, showing similar affinities of native SSB and these two mutant proteins. Thus, deletion of amino acid residues 116−177 did not severely affect the ssDNA binding ability. These results were consistent with the report obtained by fluorescence spectrometry (Williams et al., 1983; Curth et al., 1996) that the C-terminal third (16−177 residues) is not essential for ss DNA binding.

However, further deletion in C-terminal region, as seen in the panel of SSB1−105 in Figure 2, caused a dramatic decrease in the binding affinity. Half of the DNA remained free at [SSB]/[ssDNA] = 128. It is convenient to define relative binding affinities of mutant proteins and native SSB for ssDNA as the reciprocal of the molar ratio of SSB to ssDNA at which the amounts of bound and unbound ssDNA were equal. Here, the relative binding affinity of native SSB is taken to be unity. Table 1 shows that the affinity of SSB1−105 for $d(T)_{32}$ decreases by about 50-fold from the level of native SSB. Consistently for SSB1−88, although a faint band due to the protein−DNA complex appeared at [SSB]/[ssDNA] = 256, a large fraction of free $d(T)_{32}$ remained at [SSB]/[ssDNA] = 1024 where SSB1−88 seemed to be aggregated (data not shown), indicating very weak affinity of SSB1−88 for the ssDNA. Thus, the region of residues 89−115 plays a crucial role in DNA binding ability of SSB.

The N-terminal deletion mutant proteins retarded half of the free $d(T)_{32}$ at [SSB]/[ssDNA] = 512 (the panel of SSB12−177 of Figure 2), showing that the affinity of SSB12−177 for $d(T)_{32}$ was about 200-fold smaller than that of native SSB (Table 1). Thus, the deletion of N-terminal

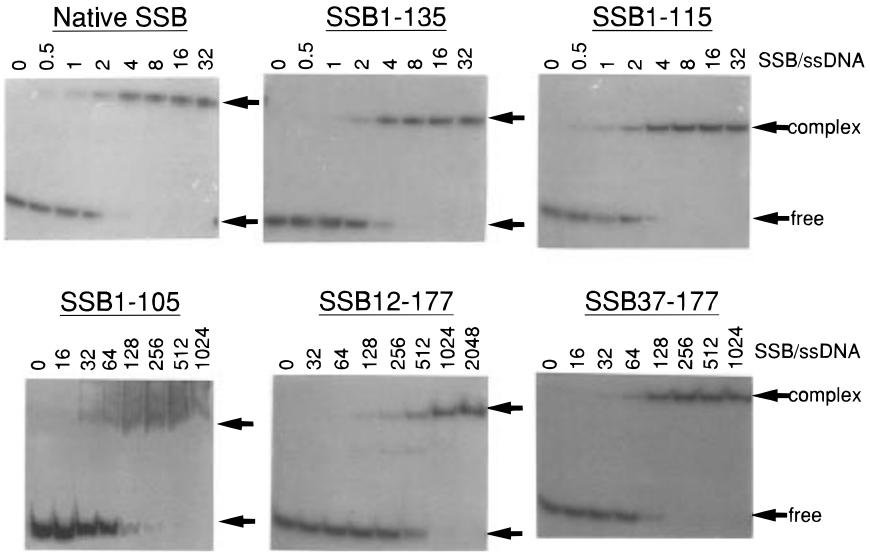

FIGURE 2: Gel mobility shift assay for $d(T)_{32}$ with native and deletion mutant SSB proteins. Numbers at the top of each lane represent the molar ratio of SSB monomer to ssDNA. ssDNA concentration was fixed to be 1.0×10^{-8} M. Experimental details are described in Materials and Methods.

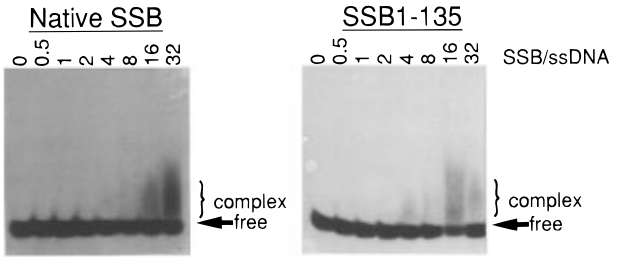

FIGURE 3: Gel mobility shift assay for single-stranded $d(T)_{16}$ with native SSB and SSB1−135. Experimental conditions were the same as in Figure 2.

11 amino acid residues severely affected the ssDNA binding ability compared to the deletion of the last 62 residues in the C-terminal. As seen in the panel of SSB37−177 of Figure 2, half of the $d(T)_{32}$ was retarded at [SSB]/[ssDNA] between 64 and 128, and the relative affinity was estimated to be about 1/50 relative to that of native SSB (Table 1). Interestingly, the further deletion of the N-terminal region up to Thr-36 caused an increase of the affinity for ssDNA. The relative binding affinity of SSB37−115, the mutant with both N-terminal and C-terminal deletions, was estimated to be about 1/200, and the binding of SSB37−105 to $d(T)_{32}$ was not detected under the conditions employed (Table 1). This result also suggested that the region of residues 106−115 is important for ssDNA binding.

To investigate the binding modes of SSB, we determined the effect of oligonucleotide length on the binding affinity of SSB deletion mutants using $d(T)_{16}$, $d(T)_{32}$, and $d(T)_{64}$. Figure 3 shows the gel mobility shift assay for native SSB and SSB1−135 to $d(T)_{16}$. The bands of $d(T)_{16}$ were not completely shifted even at [SSB]/[ssDNA] = 32, indicating small affinity for $d(T)_{16}$. Furthermore, the band of DNA−protein complex was smeared for both native SSB and SSB1−135, indicating a rapid dissociation of the complex. A similar result was obtained for SSB1−115 (data not shown). This is consistent with the results of Krauss et al. (1981) and Bujalowski and Lohman (1989a) that interaction of SSB with $d(T)_{16}$ is weak compared to that with longer $d(T)_{30-40}$, as shown by fluorescence titrations. These results implied that a certain length of ssDNA longer than 16 nucleotides was required to form a stable SSB−DNA complex.

Figure 4 shows gel mobility shift assay for native SSB, SSB1−135, SSB1−115, and SSB37−177 to $d(T)_{64}$. As seen in the panel of native SSB, about a half of the amount of $d(T)_{64}$ was retarded at [SSB]/[ssDNA] = 4, and the complete retardation occurred at [SSB]/[ssDNA] = 8. Thus, the minimum amount of protein required for the complete retardation of $d(T)_{64}$ was about twice as much as for $d(T)_{32}$ (see Figure 2). This was also true for the binding affinities of SSB1−135 and SSB1−115 for $d(T)_{64}$ and $d(T)_{32}$. This implied that the stoichiometry of the SSB−DNA complex is different, depending upon the length of oligonucleotides. Notably, two stable complexes of SSB−ssDNA, corresponding to two retarded bands (indicated as complexes **I** and **II** in Figure 4) were formed for native SSB, SSB1−135, SSB1−115, and SSB37−177 with $d(T)_{64}$. It can be simply interpreted that at lower concentrations of native SSB, one molecule of native SSB tetramer binds to one molecule of $d(T)_{64}$, forming complex **I**, whereas two molecules of SSB tetramer bind to one molecule of $d(T)_{64}$, forming complex **II**. Such two complexes may represent two distinct binding modes of SSB rather than solely different stoichiometry as will be discussed later. Thus, the deletion of residues 1−36 or 116−177 did not affect the binding mode of SSB. In contrast, SSB1−88, SSB12−177, and SSB37−115 formed only one type of the complex with $d(T)_{64}$ (data not shown). This would be only due to affinities too small to form complex **II**.

CD Spectra of Native SSB and Its Deletion Mutants Complexed with $d(T)_{32}$

CD spectra of the complex of $d(T)_{32}$ titrated with native SSB, SSB1−135, or SSB1−115 were investigated (data not shown). Increasing the amount of native SSB resulted in decreasing intensity of the CD of $d(T)_{32}$ in the range from 240 to 300 nm, producing a change of $d(T)_{32}$ conformation upon SSB binding. This result is consistent with the previous reports (Anderson & Coleman, 1975; Kuil et al. 1990) that the intensity of the CD spectra of single-stranded polynucleotide and natural DNA is decreased upon binding to SSB. Similar profiles of CD spectra were observed in the titration of $d(T)_{32}$ with SSB1−115 and SSB1−135, suggesting that SSB1−115 and SSB1−135 interact with $d(T)_{32}$ in the same way as in the case of native SSB, *i.e.*, the deletion of the C-terminal residues up to 116 does not affect the wrapping of SSB molecule by ssDNA.

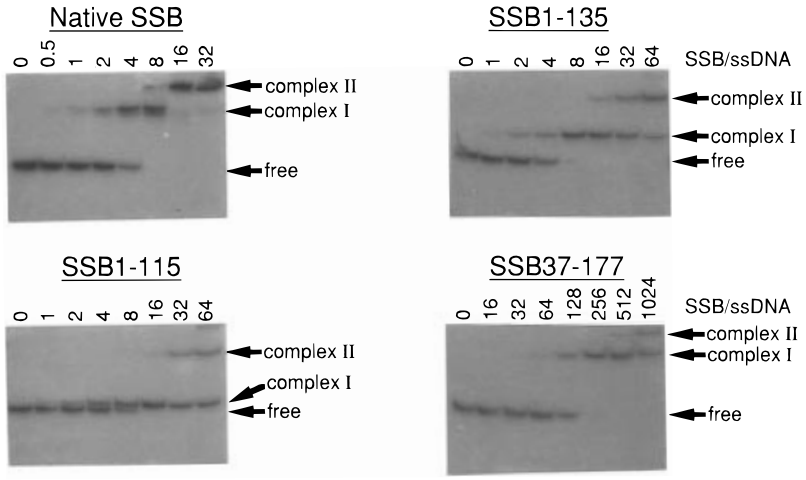

FIGURE 4: Gel mobility shift assay for single-stranded $d(T)_{64}$ with native and deletion mutant SSB proteins. Experimental conditions were the same as in Figure 2. Two retarded bands indicated as complexes **I** and **II** are seen in each panel.

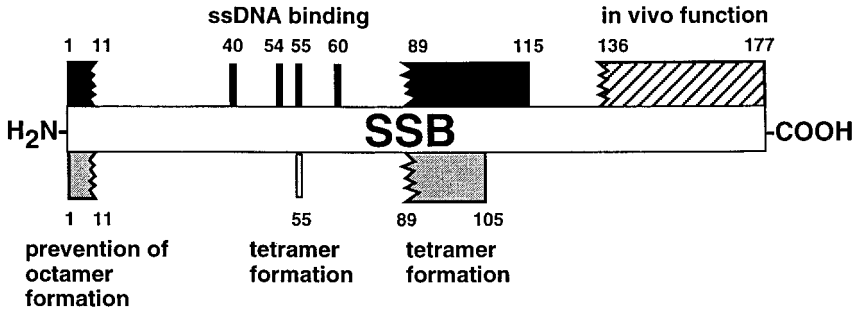

FIGURE 5: Functional dissection of SSB referred to as the abilities for DNA binding and multimerization of deletion mutant proteins. At the top, black boxes represent the region involved in ssDNA binding, and the shaded box represents the region important for *in vivo* function of SSB. At the bottom, gray boxes represent regions essential for intermolecular interaction to form multimers. Bars on both sides indicate amino acid residues revealed to be involved in ssDNA binding or tetramer formation from characterization of amino acid substitution mutant proteins (Merril et al., 1984; Williams et al., 1984; Casas-Finet et al., 1987; Khamis et al., 1987; Bujalowski & Lohman, 1991a,b).

DISCUSSION

We have examined the roles of N- and C-terminal regions of SSB in terms of the abilities of DNA binding and self-oligomerization, and the results obtained are illustrated in the functional map of SSB monomer in Figure 5. None of common motifs corresponding to the SSB domains was found, judging from a search for amino acid sequence motifs.

As described in the Results, C-terminal deletion up to the 106th amino acid residue did not affect tetramer formation of SSB (Table 1), and hence a critical region for tetramer formation was attributed to amino acid residues 89−105 (Figure 5). On the other hand, the N-terminal deletion mutants, SSB12−177 and SSB37−177 exist as octamers, suggesting that SSB has a region to control the dimerization of tetramer to form octamer. The same N-terminal deletion severely decreased the affinity for ssDNA (Table 1). This suggests a reverse correlation between octamerization and an enhancement in DNA binding. The structure of T4 gene 32 protein and a hexadeoxynucleotide (Shamoo et al., 1995) revealed that a hydrophobic pocket is included in the ssDNA binding domain of T4 gene 32 protein. The N-terminal 11 amino acids of SSB contain five hydrophobic residues. If the N-terminal hydrophobic residues are used in the ssDNA binding, the N-terminal deletion may cause an effect similar to the DNA binding, namely, octamerization upon DNA binding, as proposed by Lohman and colleagues that SSB tetramer binds cooperatively to ssDNA to form octamer in the complex (Lohman & Bujalowski, 1990; Ferrari et al., 1994; Lohman et al., 1986). Alternatively, if the N-terminal hydrophobic residues are a part of the hydrophobic core determining the general folding of the protein, the N-terminal deletion may cause a drastic change in the general structure of SSB, leading to loss of the affinity for DNA and exposure of a hydrophobic surface to promote octamerization.

Williams et al. (1984) showed that the SSB-1 mutant protein (His-55 to Tyr) exist as monomer at relatively lower protein concentrations. Therefore, oligomer formation is attributed to at least three distinct regions: residues 1−11 and 89−105 and near the His-55 residue (see Figure 5).

As seen in Table 1, there is a considerable difference in the relative affinities for $d(T)_{32}$ between SSB1−115 and SSB1−105 (1/2 *versus* 1/50). Further deletion of C-terminal region (89−106) caused a remarkable reduction of ssDNA affinity. It is worth noting that the important regions for both ssDNA binding and multimerization are overlapped in the functional map of SSB (black boxes *versus* gray boxes in Figure 5). Moreover, the substitution of His-55 to Tyr reduced the stability of the tetramer with respect to the monomer and thereby lowered the binding affinity (Williams et al., 1984; Bujalowski et al., 1991). Taken together, the formation of the tetramer is a critical factor of the high affinity for ssDNA, suggesting that the ssDNA binding domains may be formed on the surface of the tetramer and/or at the interface between SSB subunits.

Site-directed mutagenesis and spectroscopic studies showed that Trp-40 and Trp-54 are important for high-affinity binding to ssDNA, whereas Trp-88 and Trp-135 are not (Casas-Finet et al., 1987b; Khamis et al., 1987a,b). UV cross−linking of SSB to the $d(T)_8$ was found to occur almost exclusively at Phe-60 (Merril et al., 1984). Taken together, the regions important for ssDNA binding were mapped as shown in the black boxes in Figure 5.

It was shown that the C-terminal deletion up to the 116th residue does not significantly affect either the ssDNA binding affinity or multimer formation. To evaluate the function of this region *in vivo*, we introduced plasmids harboring the genes of SSB1−88, SSB1−115, and SSB1−135 into an *E. coli* strain that carries the wild type *ssb* gene under regulatable promoter. In spite of the high expression of these mutant proteins, the cells were not viable if expression from the wild type *ssb* gene was shut off (data not shown). This result indicates that the C-terminal region is important for the function of SSB *in vivo*, and this region might be involved in the interaction with other proteins in DNA metabolism (see the shaded box in Figure 5). This idea is consistent with properties of the *ssb-113* mutation (Pro-176 to Ser), which exhibits a temperature sensitive defect in replication (Vales et al., 1980). Since there does not seem to be any major change in ssDNA binding properties of SSB-113 protein compared to wild type SSB, it has been postulated that the defect in the SSB-113 protein may result from the perturbation of interactions with other replication proteins [Chase et al., 1985; see reviews by Meyer and Laine (1990), Lohman and Bujalowski (1990), and Lohman and Ferrari (1994)]. Quite recently, Curth et al. (1996) have reported that mutant SSB proteins lacking the last 10 amino acids are unable to substitute wild type SSB, indicating that the C-terminal region is involved in protein−protein interaction in the *E. coli* cells.

We observed two species of SSB−DNA complexes with $d(T)_{64}$ and one with $d(T)_{32}$ by the gel mobility shift assay. It is explained that the complexes **I** and **II** were formed with molar ratios of $d(T)_{64}$/SSB tetramer of 1:1 and 1:2, respectively. Lohman's group identified as many as three binding

modes processing site sizes of approximately 35, 56, and 65 nucleotides per tetramer under various conditions [see review by Lohman and Bujalowski (1990)]. The site size of ssDNA varies between 35 ± 5 and 65 ± 5 nucleotides per tetramer, depending on the solution conditions, particularly on the salt concentration and type. Furthermore, fluorescence titrations showed that the $(SSB)_{35}$ and $(SSB)_{65}$ modes are favored at high and low SSB binding densities, respectively, *i.e.*, one SSB molecule can bind all 65 nucleotides at large excess of binding sites, while two SSB molecules share 65 nucleotides with an excess of protein over binding sites (Bujalowski et al., 1986; Ferrari et al., 1994). The transition between the $(SSB)_{35}$ and $(SSB)_{65}$ modes is reversible at high density (Lohman et al., 1985). Thus, complexes **I** and **II** as seen in Figure 4 are most likely to be in the binding modes of $(SSB)_{65}$ [or $(SSB)_{56}$] and $(SSB)_{35}$, respectively. Herein, our results clearly show that the transition of the binding modes is induced by changing the SSB concentration; *i.e.*, the $(SSB)_{65}$ and $(SSB)_{35}$ binding modes occur at lower and higher SSB concentrations, respectively. The change of the binding modes, which may be used selectively in DNA replication and recombination as proposed previously (Lohman & Bujalowski, 1990; Ferrari et al., 1994), would be governed by the local concentrations of SSB in the cells.

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