FOR THE RECORD

The C-terminal domain of full-length *E. coli* SSB is disordered even when bound to DNA

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Abstract

The crystal structure of full-length homotetrameric single-stranded DNA (ssDNA)-binding protein from *Escherichia coli* (SSB) has been determined to 3.3 Å resolution and reveals that the entire C-terminal domain is disordered even in the presence of ssDNA. To our knowledge, this is the first experimental evidence that the C-terminal domain of SSB may be inherently disordered. The N-terminal DNA-binding domain of the protein is well ordered and is virtually indistinguishable from the previously determined structure of the chymotryptic fragment of SSB (SSBc) in complex with ssDNA. The absence of observable interactions with the core protein and the crystal packing of SSB together suggest that the disordered C-terminal domains likely extend laterally away from the DNA-binding domains, which may facilitate interactions with components of the replication machinery in vivo. The structure also reveals the conservation of molecular contacts between successive tetramers mediated by the L₄₅ loops as seen in two other crystal forms of SSBc, suggesting a possible functional relevance of this interaction.

Keywords: SSB; crystal structure; DNA replication; ssDNA; protein disorder

The *Escherichia coli* single-stranded DNA (ssDNA)-binding protein (SSB) plays essential roles in DNA replication, recombination, and repair by stabilizing ssDNA intermediates that are generated during DNA processing (Chase and Williams 1986; Meyer and Laine 1990; Lohman and Ferrari 1994). *E. coli* SSB protein assembles as a stable homotetramer and can bind long ssDNA in two major binding modes, referred to as (SSB)₃₅ and (SSB)₆₅, where the subscripts reflect the average number of ssDNA nucleotides

occluded by the SSB tetramer (Lohman and Overman 1985). In the (SSB)₃₅ mode, an average of two subunits of the stable tetramer interact with ssDNA, whereas in the (SSB)₆₅ mode, the ssDNA wraps around the tetramer, interacting with all four subunits (Lohman and Overman 1985; Bujalowski and Lohman 1986; Lohman and Ferrari 1994). The intertetramer positive cooperativity upon ssDNA binding of SSB also differs for these two binding modes, with the fully wrapped (SSB)₆₅ mode displaying only moderate “limited” cooperativity, whereas the (SSB)₃₅ mode displays an “unlimited” positive cooperativity, which results in the “classic” formation of clusters of protein along the ssDNA (Chrysogelos and Griffith 1982; Griffith et al. 1984; Lohman et al. 1986b). The transition between these two modes is influenced by monovalent and divalent salt concentration as well as the protein-to-DNA ratio. Thus, the ssDNA-binding properties of the *E. coli* SSB tetramer, and other SSB proteins in this class, differ from those of either the phage T4 gene 32 protein or the eukaryotic heterotrimeric RPA protein.

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Recent structural analyses of a chymotryptic fragment of *E. coli* SSB (SSBc) containing the ssDNA-binding domain of the protein (residues 1–135), which still forms a tetramer, have provided a detailed view of the structural determinants underlying its interactions with ssDNA (Ragunathan et al. 1997, 2000; Yang et al. 1997). These studies revealed the details of the topology for the wrapping of ssDNA around the tetrameric SSBc scaffold, and the fact that consecutive SSBc tetramers participate in crystal packing interactions via their respective L_{45} loops (loops between the β_4 and β_5 strands in the SSBc OB fold), resulting in linear SSBc polymers. Taken together, these observations suggest that the interactions mediated by the L_{45} loops may play a role in facilitating the unlimited positive cooperativity observed for the (SSB)₃₅ binding mode.

In addition to the interactions of SSB with ssDNA, a second essential aspect of its function is its interaction with proteins involved in DNA metabolism. So far, a number of proteins have been demonstrated to interact with SSB: exonuclease I (Genschel et al. 2000), RecO (Umezumi and Kolodner 1994; Kantake et al. 2002), uracil DNA glycosylase (Handa et al. 2001), and the χ subunit of DNA polymerase III (pol III) holoenzyme (Glover and McHenry 1998; Kelman et al. 1998; Witte et al. 2003). An important consensus that has emerged from these studies is that SSB uses its C-terminal domain to interact with these protein partners. More detailed studies of the binding of SSB to the χ subunit of the pol III holoenzyme have shown that the cornerstone of this interaction lies within the last 26 amino acid residues from the C terminus of SSB (Kelman et al. 1998; Witte et al. 2003).

Although the structure and interactions of the DNA-binding domain of SSB have been described in detail (Ragunathan et al. 1997, 2000; Yang et al. 1997), the structure of the C-terminal domain (residues 113–177) has remained elusive. Here, we attempt to shed light onto the structural features of full-length SSB and how these may relate to its function.

Results and Discussion

Previously, full-length SSB protein (SSB) was crystallized in the absence of DNA (apoSSB; Ollis et al. 1983). However, the structure of apoSSB was never determined, and it subsequently became apparent that the crystalline form of apoSSB contained a mixture of proteolytic fragments of the full-length protein. In undertaking structural studies of SSB in complex with ssDNA, we ensured prior to data collection that the SSB protein was fully intact and that ssDNA was indeed present in crystals of the complex (Fig. 1).

The structure of tetrameric SSB in complex with dC(pC)₃₄ was determined by multiwavelength anomalous diffraction (MAD) and was refined to 3.3 Å (Fig. 2A; Table 1). The core DNA-binding domain of SSB could be readily

Figure 1. Biochemical characterization of SSB-ssDNA crystals. (A) SDS-PAGE analysis of the protein in crystals of the SSB-dC(pC)₃₄ complex. The band corresponding to crystalline SSB-dC(pC)₃₄ complex appears a little less pronounced than the control. This is due to the inherent difficulty of controlling the loaded amount of protein when one deals with dissolved protein crystals. (B) Urea-denaturing PAGE analysis of the ssDNA in crystals of the SSB-dC(pC)₃₄ complex.

modeled in the experimental electron density maps up to residue 112 in each molecule, and it is almost identical to the structure of SSBc with and without ssDNA (Ragunathan et al. 1997, 2000). On the other hand, the electron density corresponding to the C-terminal domain was undecipherable, suggesting high mobility in this part of the structure. In contrast to previous studies of SSBc in complex with dC(pC)₃₄ (Ragunathan et al. 2000), the electron density for ssDNA was generally poor and discontinuous, and only well defined where the DNA bases are involved in stacking interactions with tryptophan residues of SSB (Ragunathan et al. 2000). Convincing modeling of the ssDNA was therefore not possible. Although the effective resolution of our analysis is a limiting 3.3 Å, the sharp contrast in electron density quality between the disordered C-terminal domain and the well-ordered core domain strongly suggests that our

Figure 2. Structure of SSB. (A) Representative region (the $\beta 1$ strand) of the experimental electron density at 3.3 Å resolution. The electron density is an excerpt from a map calculated with two-wavelength MAD phases after electron density modification by solvent flipping and is contoured at 1.5 σ . The final refined model is shown as a reference in stick representation color-coded in yellow for carbon, blue for nitrogen, and red for oxygen. (B) A view of the crystal packing of the SSB tetramer. The protomer is drawn in cyan and is surrounded by symmetry related molecules. The white asterisks indicate the points (Leu 112) in each of the four subunits in the SSB tetramer at which interpretable experimental electron density stops. The horizontal dyad axis is indicated as a white dotted line as a reference, and the cavities in the packing accommodating the disordered C-terminal regions are shown in red circles. (C) Conservation of the L_{45} -mediated interactions of successive SSB molecules to produce a string polymer of SSB molecules. This view is obtained by rotating the one in B by 90 degrees counterclockwise around the vertical axis. The L_{45} mediated interactions are encircled in dotted circles.

interpretation is qualitatively correct. Furthermore, the low-resolution diffraction of SSB-dC(pC)₃₄ crystals is consistent with the observed molecular disorder and with the consensus that macromolecules with high degrees of flexibility and/or lack of regular structure either fail to crystallize or yield weakly diffracting crystals. Iterative cycles of fourfold noncrystallographic symmetry averaging and solvent flip-

ping improved the electron density for the DNA-binding core, but not the missing C-terminal regions, suggesting that the C-terminal domains adopt several distinct conformations and/or that they are largely unstructured. The absence of specific interactions of the C-terminal regions with the core of the protein, and the presence of pronounced bulk solvent pockets next to the DNA-binding domains suggest

Table 1. Data collection and refinement

	Resolution (Å)	Reflections (total/unique)	Completeness (%)	R_{sym} (%) ^a	I/ σ (I)
Data set					
SeMet-1, 0.9879 Å	30–3.3	101,543/21,921	99.4 (100.0)	6.0 (27.3)	23.3 (4.0)
SeMet-1, 0.9879 Å, Friedel pairs merged	30–3.3	101,104/11,620	99.4 (100.0)	6.2 (29.2)	30.8 (5.9)
SeMet-2, 0.9794 Å	30–3.3	104,045/22,504	99.4 (99.7)	6.1 (29.0)	23.0 (3.7)
SeMet-3, 0.9792 Å	30–3.3	103,625/22,442	99.3 (100.0)	6.2 (27.1)	23.5 (4.1)
Refinement					
Resolution (Å)	30–3.3				
F / σ (F) cutoff	0				
Number of reflections (working set/test set)	10,143/1166				
Total number of atoms	2817				
R_{cryst} , R_{free}	0.288, 0.309				

Numbers in parentheses correspond to values in the highest resolution shell (3.4 to 3.3 Å).

^a $R_{\text{sym}} = \sum |I - \langle I \rangle| / \sum I$, where I = observed intensity, and $\langle I \rangle$ = average intensity for symmetry related reflections.

that the disordered/unstructured C-terminal regions may emanate laterally away from the core of the structure (Fig. 2B). At the same time we observe that adjacent SSB tetramers within the crystal interact via their L_{45} loops to form a “filament” of protein molecules (Fig. 2C). Such interactions have previously been observed in two different crystal forms of SSBc (Ragunathan et al. 1997, 2000). The fact that we observe this same interaction in a third distinct crystal lattice strongly suggests that this may be a general property of SSB and that it may form the structural basis for cooperative binding of SSB in its (SSB)₃₅ binding mode.

To our knowledge this is the first experimental evidence that the C-terminal domain of SSB shows such disorder, even when bound to ssDNA. Although it is disappointing that we cannot provide structural details of the C-terminal regions of SSB, we suggest that the observed disorder of this region and its likely location with respect to the core structure may have functional significance. The potential roles of intrinsically disordered/unstructured proteins in biology and disease has been noted and discussed (Wright and Dyson 1999; Tompa 2002). Therefore, our findings come at an opportune time to assist further studies aiming to more formally study the apparent inherent disorder of the C-terminal domain of SSB.

The low sequence complexity of the C-terminal domain of SSB (heavily populated by proline, glycine, and glutamine residues) had earlier hinted for the possibility of high disorder and lack of structure (Sancar et al. 1981). Indeed, predictions of disorder carried out by using disEMBL (<http://dis.embl.de>) Linding et al. 2003) identified the entire C-terminal domain as potentially unstructured/disordered. Furthermore, alignment of SSB sequences from diverse sources revealed the presence of a conserved acidic tail at the end of the C-terminal domains (Kelman et al. 1998). Interestingly, this region forms a C-terminal α -helix in the crystal structure of the phage T4 gene 32 protein and was found to lie on the surface of the molecule, suggesting that

interactions with partner proteins take place in close proximity to the core of the DNA-binding domain (Shamoo et al. 1995). The absence of such a structural feature in *E. coli* SSB suggests that interactions of SSB with proteins of the replication machinery, such as the χ subunit of Pol III holoenzyme, may take place away from the DNA-binding core of the protein. The C terminus of SSB contains the negatively charged consensus sequence DDDIPF, which may act as the tether-point onto complementary positively charged surfaces on the χ subunit. Indeed, the recently published structure of the χ - ψ heterodimeric complex of *E. coli* DNA Polymerase III has revealed a conserved, positively charged patch defined by residues 124–135 on the surface of the χ subunit at the distal end of the χ - ψ complex (Gulbis et al. 2004). It was therefore proposed that this may be the docking site for the C terminus of SSB (Gulbis et al. 2004).

Materials and methods

Crystallization of the SSB–dC(pC)34 complex

Selenomethionyl SSBfl and dC(pC)34 oligodeoxynucleotide were prepared according to established protocols (Lohman et al. 1986a; Yang et al. 1990; Wong et al. 1992). The corresponding complex was formed at a 1:2 molar ratio [tetramer SSB per dC(pC)34] in a buffer containing 100 mM Tris HCl (pH 8.5), 0.5 M NaCl, 1 mM EDTA, and 1 mM β -mercapto-ethanol at 20°C and then diluted 100-fold in 100 mM Tris HCl (pH 8.5), 1 mM EDTA, and 1 mM β -mercapto-ethanol to adjust the NaCl concentration to 5 mM. The complex was then concentrated to ~10 mg/mL. Crystals were grown in a hanging drop against a reservoir containing a solution of 100 mM Hepes (pH 7.5), 20% (v/v) PEG200, and 20% (w/v) PEG4000. Sizable crystals (0.3 × 0.3 × 0.3 mm) of SSB–dC(pC)34 appeared reproducibly within 2 to 3 months. Crystals of SSB–dC(pC)34 belong to space group P3₁12 with $a = b = 60.9$ Å, $c = 348.9$ Å, $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$ and a tetramer in the asymmetric unit.

Characterization of SSB–dC(pC)34 crystals

SSB–dC(pC)34 crystals were tested for both the presence of DNA and the integrity of the protein. To assess the integrity of the

protein, crystals were first washed by using the same procedure as above and then dissolved in an SDS-PAGE loading buffer, and the protein was visualized on a Coomassie blue-stained 12% SDS-PAGE gel (Fig. 1A). To test for the presence of DNA, the crystals of SSB-dC(pC)34 were first repeatedly washed in a stabilizing solution containing 100 mM Hepes (pH 7.5), 20% (v/v) PEG200, and 20% (w/v) PEG4000 and then dissolved in a standard Tris-borate-EDTA (TBE) buffer containing 6 M urea and 10% glycerol. The sample was then loaded onto a 19% polyacrylamide slab gel. After electrophoresis for 3 h at 5 to 10 W (constant power), the gel was stained by using a solution of 0.01% (w/v) stains-all (Aldrich) and 40% (v/v) formamide (Fig. 1B).

Structure determination

Complete data sets to 3.3 Å resolution were collected at three wavelengths (SeMet-1 through -3; Table 1) at beam line 19ID of the Structural Biology Center, Advanced Photon Source, and were processed with DENZO and SCALEPACK (Otwinowski and Minor 1997). Automated approaches in obtaining phasing information by MAD or single-wavelength anomalous diffraction (SAD) using CNS (Brünger et al. 1998) and SOLVE (Terwilliger and Beredzen 1999) failed due to the fact that the selenium substructure could not be determined. The following strategy was then successfully used to obtain de novo phasing information.

A molecular replacement solution for a tetramer in the asymmetric unit was obtained by using the structure of the chymotryptic fragment of SSB (PDB code 1EYG; Raghunathan et al. 2000). This was then combined with data collected at the absorption peak of selenium (SeMet-3; Table 1), to produce an anomalous difference Patterson map that revealed 12 selenium sites in the core domain. The additional selenium site expected at the end of the C-terminal domain could not be located in any of the four SSBfl molecules indicative of local and/or domain disorder. Introduction of the derived selenium substructure into MAD phasing protocols in CNS and SOLVE using data from all three wavelengths failed to produce an interpretable electron density. On the other hand, SAD phasing in CNS with either data collected at the selenium absorption peak (SeMet-3; Table 1) or the selenium absorption edge (SeMet-2; Table 1) followed by electron density modification by solvent flipping yielded a partially interpretable electron density map. Surprisingly, a greatly improved electron density map could be obtained by MAD phasing in CNS using both SeMet-2 and SeMet-3, followed by solvent flipping (Fig. 2A). The core domain of SSB could be readily modeled in the experimental electron density maps up to residue 112 in each molecule, whereas the electron density of the anticipated C-terminal domain was undecipherable suggesting extreme disorder. The electron density for ssDNA was also poor, and only well defined where bases are involved in stacking interactions with tryptophan residues of SSB. Iterative cycles of fourfold noncrystallographic symmetry averaging and solvent flipping improved the electron density for the DNA-binding core but not the missing C-terminal regions, suggesting that the C-terminal domains adopt distinct conformations. Modeling of the SSB beyond residue 112 and of ssDNA was therefore not possible. Initially, model building was carried out by docking the DNA-binding domain of SSB (Raghunathan et al. 2000) into experimental electron density maps by using the program O (Jones et al. 1991). Crystallographic refinement was carried out by using rigid-body refinement and conjugate gradient minimization in CNS, followed by grouped-temperature factor refinement. Heavy NCS-restraints were initially applied evenly to all four protomers but were subsequently optimized for loop regions by using the behavior of the R_{free} value as a guide.

Crystallographic coordinates and structure factors have been deposited with the Protein Data Bank (<http://www.rcsb.org>) with accession code 1SRU and will be available upon the publication date of this article.

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