Single-stranded DNA-binding protein of *Deinococcus radiodurans*: a biophysical characterization

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**ABSTRACT**

The highly conserved bacterial single-stranded DNA-binding (SSB) proteins play an important role in DNA replication, repair and recombination and are essential for the survival of the cell. They are functional as tetramers, in which four OB(oligonucleotide/oligosaccharide binding)-folds act as DNA-binding domains. The protomer of the SSB protein from the extremely radiation-resistant organism *Deinococcus radiodurans* (DraSSB) has twice the size of the other bacterial SSB proteins and contains two OB-folds. Using analytical ultracentrifugation, we could show that DraSSB forms globular dimers with some protrusions. These DraSSB dimers can interact with two molecules of *E.coli* DNA polymerase III χ subunit. In fluorescence titrations with poly(dT) DraSSB bound 47–54 nt depending on the salt concentration, and fluorescence was quenched by more than 75%. A distinct low salt binding mode as for *Eco* SSB was not observed for DraSSB. Nucleic acid binding affinity, rate constant and association mechanism are quite similar for *Eco* SSB and DraSSB. In a complementation assay in *E.coli*, DraSSB took over the in vivo function of *Eco* SSB. With DraSSB behaving almost identical to *Eco* SSB the question remains open as to why dimeric SSB proteins have evolved in the *Thermus* group of bacteria.

**INTRODUCTION**

Single-stranded DNA-binding (SSB) proteins play a vital role in the sustainment of almost every form of life we know on earth. They protect and configure the vulnerable state of unwound and single-stranded DNA (ssDNA) for efficient use by enzymes involved in DNA metabolism, such as replication, recombination and repair. The function of ssDNA binding is found in seemingly very different protein classes ranging from monomers over homodimers and homotetramers to heterooligomers. However, all SSB proteins share a structural motif called OB(oligonucleotide/oligosaccharide binding)-fold (1,2). One of these classes is formed by homotetrameric SSB proteins which play a vital role in bacteria and eukaryotic mitochondria. Their 3D structures, which have been determined for such divergent organisms as *E.coli* and human, are quite similar (3–5), and thus this class of proteins constitutes an evolutionary strictly conserved structural principle.

In light of this structural conservation, it was surprising when in silico analysis of the *ssb* genes from the bacterial *Thermus* group suggested the proteins encoded by these genes to belong to the class of homotetrameric SSBs, although they were about twice the size compared with other proteins of this class. The protomers of *Thermus* SSBs contain two OB-folds per monomer, and therefore it was not surprising that these proteins form homodimers (6). These homodimers thus mimic the homotetrameric SSBs.

One might argue that the origin of homodimeric SSB proteins was a duplication of the gene of the homotetrameric SSB proteins, and there have been some speculations about evolutionary raison d’être for such a duplication. One of these speculations puts an evolutionary advantage to the fact that the two OB-folds in the homodimeric SSB can evolve separately and thus can help in surviving the hostile environments *Thermus* bacteria live in (7).

Recently, several homodimeric SSBs from different *Thermus* species have been cloned and isolated as proteins (8,9) and for the SSB from *Deinococcus radiodurans* (DraSSB) a 3D structure has been determined by X-ray crystallography (7). The overall structure of DraSSB in its OB-folds is quite similar to the human mitochondrial and *E.coli* SSB structure.

However, owing to the lack of solid biochemical and biophysical data on the solution properties of DraSSB, the interpretation of structural details remains speculative. We, therefore, have isolated the gene for this protein, expressed it in *E.coli* and purified it to homogeneity. In this work, we will describe the biophysical and biochemical characterization of

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the protein itself and its protein–protein and protein–DNA interactions. As in the structure, the overall properties of DraSSB resemble those of the homotetrameric SSBs albeit with some differences in detail.

**MATERIALS AND METHODS**

**Buffers and reagents**

*D.radiodurans* R1 strain DSM20539 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Germany). Poly(dT) (~1400 nt in length), poly(rU) and poly(dA–dT) were purchased from Amersham Biosciences. Polynucleotide concentrations are given in monomer residues throughout the text and were determined spectrophotometrically using absorption coefficients of 8600 M$^{-1}$ cm$^{-1}$ for poly(dT) at maximum (10) and 9200 M$^{-1}$ cm$^{-1}$ and 6700 M$^{-1}$ cm$^{-1}$ at 260 nm for poly(rU) and poly(dA–dT), respectively (11).

Protein concentrations were determined using the absorption coefficients at 280 nm calculated from amino acid composition (12): 29 400 M$^{-1}$ cm$^{-1}$ for χ subunit of *E.coli* DNA polymerase III, 82 000 M$^{-1}$ cm$^{-1}$ for DraSSB dimer and 113 000 M$^{-1}$ cm$^{-1}$ for EcoSSB tetramer. DraSSB and EcoSSB concentrations are given in dimers and tetramers, respectively, throughout the text.

All experiments were carried out in potassium phosphate buffer at pH 7.4 (KP$_1$) containing NaCl as neutral salt. Concentrations are given at the respective experiments.

**Cloning of D.radiodurans ssb gene**

The *ssb* gene was amplified from heat disrupted *D.radiodurans* cells by PCR. Primers used were 5′-GGAGGACCATGGCCC-GAGGCCATGAACCA-3′ for forward and 5′-GAAGAGGAT-CCTCATTGGGTGTCTTTGTTG-3′ for reverse priming. Primers were selected to include start and stop codon containing sequences (italics) of the *DraSSB* gene accession no. AJ564860 (*vide infra*). PCR was carried out using Pfu DNA polymerase (Stratagene), 200 μM dNTP and 0.5 μM of each primer with an annealing temperature of 53°C and 30 cycles. The PCR product was purified using a PCR purification kit (Qiagen). The amplified product was digested with NcoI and BamHI and cloned into the NcoI and BamHI sites of pET15b (Novagen) to generate pET15b-DraSSB. This plasmid was used to transform the *E.coli* strain LK111. The procedure was repeated using a different lot of the *D.radiodurans* strain DSM20539. Different clones were selected and the *DraSSB* gene was completely sequenced in both strands (MWG Biotech) for both lots of *D.radiodurans*.

**Expression and purification of SSB protein from D.radiodurans**

For protein expression, the plasmid pET15b-DraSSB was used to transform the *E.coli* strain BL21(DE3) pLysS. A volume of 900 ml of an overnight culture grown in the presence of 100 μg/ml ampicillin and 30 μg/ml chloramphenicol at 30°C was used to inoculate 10 l of standard Luria–Bertani (LB) medium and the cells were grown at 30°C. An aliquot of 100 ml of the overnight culture was used for plasmid preparation (Qiagen) and subsequent DNA sequencing to confirm the sequence of the expressed gene shortly before induction. Protein expression was induced after growing the cells to A$_{600}$ nm = 1.0 by the addition of 1 mM isopropyl-β-d-thiogalactopyranoside and was continued for 4 h. After cell harvesting and washing with buffer W [0.2 M NaCl, 50 mM Tris–HCl, pH 8.3, 1 mM EDTA and 10% (w/v) sucrose], the pellet was resuspended in buffer S (buffer W containing 15 mM spermidin), frozen and stored at −70°C.

When cells were thawed, one tablet of ‘complete EDTA-free’ protease inhibitor (Roche) per 50 ml and 0.04% sodium-deoxycholate were added. After sonication and removing of cell debris by preparative ultracentrifugation, proteins were precipitated by the addition of polyethyleneimine, pH 6.9 (final concentration 0.4%) for 20 min at 4°C and pelleted. The pellet was extracted with TGE-400 buffer [0.4 M NaCl, 50 mM Tris–HCl, pH 8.3, 1 mM EDTA and 20% (w/v) glycerol] and insoluble material was removed by centrifugation. The clear supernatant was precipitated with 20% (w/v) (NH$_4$)$_2$SO$_4$ overnight at 4°C. The pellet was redissolved in TGE400 and loaded on a BlueSepharose column (13) equilibrated in the same buffer. After washing the column with TGE400, a linear salt gradient (0.4–3 M NaCl in the same buffer) was used to elute the protein. Fractions were analyzed by SDS–PAGE (14), pooled and precipitated with 20% (w/v) (NH$_4$)$_2$SO$_4$. The pellet was redissolved in SBP buffer [0.5 M NaCl, 20 mM KP$_1$, pH 7.4, 1 mM EDTA and 20% (w/v) glycerol] and dialyzed against the same buffer containing 1 M NaCl and 60% (v/v) glycerol and stored at −20°C. The protein preparation was at least 97% pure as judged from Comassie Brilliant Blue stained SDS gel.

**Complementation assay**

In *E.coli* strain RDP268, the chromosomal SSB gene is replaced by a kanamycin resistance and the essential SSB protein is encoded by the plasmid pACYCssb (15). This plasmid confers chloramphenicol resistance to the cells. pACYCssb is essential for the survival of the cells and can be replaced by another plasmid, only if it contains a gene whose product can take over *EcoSSB* function in vivo. We used the strain RDP268 to transform the pSF1 (16) derivative pSF1-DraSSB, which carries the *DraSSB* gene under the control of the λ _p_ 1 promoter and confers ampicillin resistance. In RDP268 (pSF1-DraSSB), the _p_ 1 promoter is not repressed leading to a high constitutive *DraSSB* expression. The cells were grown in the presence of 100 μg/ml ampicillin and 5 μg/ml kanamycin but omitting chloramphenicol. After four subsequent inoculations of 4 ml LB medium, allowing the cells to grow for 24 h at 37°C, 100–200 cells were plated on LB medium containing 100 μg/ml ampicillin and 5 μg/ml kanamycin. Clones that lost the plasmid pACYCssb were identified by replica plating on LB medium containing 30 μg/ml chloramphenicol and 5 μg/ml kanamycin. SSB proteins in chloramphenicol-sensitive clones were characterized by western blot analysis.

**Western blot analysis**

SSB proteins blotted onto a PVDF membrane were detected immunologically by a polyclonal anti-*EcoSSB* serum from rabbit. Rabbit antibodies were marked by alkaline phosphatase-conjugated goat anti-rabbit antibodies and detected by alkaline
phosphatase chemiluminescence reaction (CDP-Star, Tropix). Exposed X-ray films were digitized.

Analytical ultracentrifugation

Analytical ultracentrifugation was performed in a Beckman XL-A analytical ultracentrifuge using an An50Ti or An60Ti rotor at 20°C. Concentration profiles were measured with UV absorption scanning optics of the centrifuge.

Sedimentation velocity

Sedimentation velocity experiments were carried out in double-sector centerpieces at 20 000–60 000 r.p.m. Sedimentation rate constants were obtained by analyzing the movement of the sedimenting boundary or by fitting a numerical solution of Lamm’s differential equation (17) to the concentration profiles using the BPCFIT software package (18). For analysis of interactions, data were evaluated with SEDFIT (19) to yield diffusion-corrected differential sedimentation coefficient distributions c(s). The areas under separate peaks of such a distribution are a measure of the absorption of the differently sedimenting components and can be analyzed to yield a binding isotherm (20). For hydrodynamic analysis, s-values were corrected to \( s_{20\text{w}} \). Since the partial specific volume of complexes of different macromolecules with unknown stoichiometry cannot be calculated, such a correction could not be performed in these cases and uncorrected s-values had to be used.

Sedimentation equilibrium

Sedimentation equilibrium experiments for molar mass determination were carried out with 120 μl samples in 6-channel-centerpieces in an An50Ti rotor running at 20°C at 14 000 r.p.m. until no change in concentration distribution could be observed for at least 12 h. Scans from these 12 h were averaged and apparent molar masses were evaluated by fitting the concentration gradient with

\[
A(x) = A_{\text{offset}} + A(x_0) \cdot e^{-\frac{d\rho}{\omega_0 \cdot \rho^2} (x-x_0^2)}
\]

where \( x \) is the distance from centre of rotation, \( \omega \) is the angular velocity of the rotor, \( v \) is the partial specific volume of the solute calculated from amino acid composition (21), \( \rho \) is the density of the solution, \( A(x) \) is the absorption at position \( x \), \( A(x_0) \) is the absorption of the macromolecule at \( x_0 \) and \( A_{\text{offset}} \) is the absorption of the buffer measured near the meniscus after sedimenting the protein for 8 h at 44 000 r.p.m. Fitting was carried out with BPCFIT (18). Protein stability against denaturation by guanidine hydrochloride (GuaHCl) was measured in sedimentation equilibrium experiments using different concentrations of GuaHCl. Note that guanidinium denatured proteins have a molar mass somewhat higher than the molar mass of the covalent protein chain owing to preferential binding of the denaturant.

DNA melting curves

DNA melting curves were measured in a DMR10 (Zeiss, Germany) spectrophotometer as described previously (22) using a heating rate of 20 K/h. The experiments were carried out with 38 μM nucleotides of poly(dA–dT) in 75 mM NaCl, 20 mM KP. As long as the temperature did not exceed 50°C, no significant differences between heating and cooling curves could be observed, confirming the reversibility of melting. Above 50°C, irreversible heat denaturation of DraSSB occurred.

Fluorescence titrations

Fluorescence titrations were carried out in a Schoeffel RRS1000 spectrofluorimeter as described previously (23). Excitation wavelength was 295 nm and emission was detected at 350 nm. To avoid inner filter effects, concentrations of protein and single-stranded nucleic acid were chosen such that the total absorption of the solution did not exceed 0.05 throughout the titration. After each addition, the solution was allowed to equilibrate between 60 and 600 s until no fluorescence change could be observed any longer. Binding curve analysis was carried out using the model of Schwarz and Watanabe (24) with \( n \) as binding site size, \( \omega \cdot K \) as cooperative binding affinity and fluorescence quench \( Q_t \) as parameters. Fluorescence quench is defined as \( 1 - F_{\text{bound}}/F_{\text{free}} \), where \( F_{\text{free}} \) and \( F_{\text{bound}} \) denote the fluorescence intensities measured for free and nucleic acid bound protein, respectively.

Stopped-flow kinetics

Stopped flow kinetics for the determination of the association rate constant of DraSSB and poly(dT) was measured with an Applied Photophysics π+ system using tryptophan fluorescence excited at 295 nm and observed through a WG320 filter. Several traces collected for each concentration were accumulated to reduce noise. When analyzing the binding of SSB proteins to long ssDNA, a simple bimolecular model is insufficient, since one has to consider the length distribution of gaps between protein covered sites. At intermediate saturation, there will be gaps between bound proteins too short to accommodate binding of further ligands. A thermodynamic equilibrium model for such a binding of a multidentate ligand to a long linear polymer (24) has been extended to a kinetic model (10,25). In this model, it is assumed that in the rate-limiting bimolecular step of association, SSB forms an initial stable encounter complex with a shorter binding site length and subsequently redistributes in a monomolecular manner on the ssDNA. Data measured at different concentrations were analyzed by globally fitting the parameters of this kinetic model using the program BPCFIT (18).

RESULTS

Protein sequence

We amplified the postulated reading frame for *D. radiodurans* SSB protein from the strain DSM20539. Sequencing of the PCR product yielded an open reading frame (ORF) coding for 301 amino acids. The sequence was at variance with the genomic sequence published (EMBL accession no. AE001873, gene DR0099), which obviously contained sequencing errors leading to frameshifts and an early termination sequence. The same discrepancy was also found recently by others (9). The corrected DraSSB sequence was deposited in the EMBL database [accession numbers AJ564860 (this paper) and AJ293617 (9)]. The ORF shows a large sequence similarity to other SSB proteins, such as SSBS from *Thermus aquaticus*
and Thermus thermophilus (6,8,9). Comparing the DraSSB sequence with other bacterial SSB sequences [e.g. from E.coli (26)] shows that the N-terminal DNA-binding domain (the OB-fold) occurs twice in tandem in DraSSB and the other Thermus SSBs while it occurs only once in the EcoSSB gene. A possible explanation for this difference is a gene duplication event that occurred during the evolution of the Thermus group SSBs. The highly conserved acidic C-terminus with the consensus sequence DDDIPF is also present in DraSSB (EDDLLPF).

Structural characterization of DraSSB

DraSSB protein overexpressed in E.coli was purified to homogeneity (>97%). Figure 1 shows a sedimentation velocity experiment in the analytical ultracentrifuge. Analysis of the sedimenting boundary using Lamm’s differential equation yields a sedimentation rate constant of $s_{20,w} = 3.95S$ and a diffusion constant of $D_{20,w} = 6.3 \times 10^{-11} \text{ m}^2 \text{s}^{-1}$. Combining these two values with the partial specific volume of $7.32 \times 10^{-4} \text{ m}^3 \text{kg}^{-1}$ calculated from the amino acid composition (21) gives a molar mass of 55 kg/mol. The molar mass of the covalent protein chain of DraSSB is calculated to be 32.7 kg/mol. Since the diffusion coefficient evaluated from the broadening of the sedimenting boundary tends to be overestimated owing to artificial broadening effects (27), a molar mass derived from sedimentation and diffusion coefficients constitutes a lower limit and the finding, thus, is a clear indication that the native state of DraSSB is a homodimer. Using the molar mass of the dimer as calculated from the amino acid composition, the sedimentation rate constant of 3.95S leads to a frictional ratio of 1.5. Since for globular hydrated proteins this frictional ratio is expected to be 1.1–1.2 (27), a value of 1.5 shows the protein to be either strongly asymmetric or globular with several protuberances. For EcoSSB, it has been shown that the large frictional coefficient of 1.42 is reduced to 1.25 when cleaving off the C-terminal third of the protein (11). Thus, it seems likely that similar to EcoSSB the C-terminal part of DraSSB extends into the solution. This is also supported by gel filtration experiments, which suggest DraSSB to be asymmetric (9), and by recent X-ray crystallographic studies where the C-terminal part of DraSSB could not be localized (7), probably owing to large disorder.

Sedimentation equilibrium in the analytical ultracentrifuge gave a molar mass of $M = 62 \pm 2 \text{ kg/mol}$ with no indication of multiple species or aggregation, confirming the result obtained for a differently prepared DraSSB before (9) and clearly showing again that DraSSB forms homodimers.

We have tested the conformational stability of DraSSB by sedimentation equilibrium experiments in the presence of different concentrations of GuHCl. GuHCl denaturation, monitored by changes in the apparent molar mass (Figure 2), results in a transition midpoint at 1 M denaturant. DraSSB, therefore, is clearly more sensitive to GuHCl-induced denaturation than EcoSSB whose transition midpoint is at 1.7 M (Figure 2). Thus, the folded state of DraSSB is less stable than the bacterial prototype EcoSSB. This relative instability is also reflected in the temperature stability of these two proteins. While DNA melting experiments with EcoSSB can be performed up to 65°C, DraSSB irreversibly denatures at temperatures above 50°C.

In vivo detection of SSB in D.radiodurans

It remains to be demonstrated that the ORF cloned and expressed in E.coli encodes a genuine protein of D.radiodurans. Western blot analysis shows that a polyclonal anti-EcoSSB serum cross-reacts with cloned DraSSB and detects a correctly sized protein in a total protein extract from D.radiodurans (Figure 3A).

DraSSB can replace EcoSSB in vivo

Though different in oligomerization, EcoSSB and DraSSB share the ssDNA-binding folds (OB-fold) (7) and the C-terminal region, for which important protein–protein interactions have been shown (28–30). Therefore, we tested whether DraSSB can take over the EcoSSB function in vivo using an ssb deletion strain (RDP268). In RDP268, the chromosomal ssb gene has been replaced by a kanamycin resistance and the vital SSB function is supplied by EcoSSB encoded on the chloramphenicol resistance conferring plasmid pACYCssb (15). After transformation of these cells using pSF1-DraSSB,
which encodes resistance against ampicillin, and subsequent
inoculations (cf. Materials and Methods), we could isolate
clones that showed resistance against ampicillin and kanamyc-
bin but not against chloramphenicol. These clones must have
lost the pACYC184 plasmid encoding for EcoSSB. SSB
expression was examined by western blot analysis using an
anti-EcoSSB serum as a primary antibody (Figure 3B). While
in RDP268 EcoSSB could be detected, the chloramphenicol-
sensitive clones showed an expression of only DraSSB
(32.7 kDa) but not of EcoSSB (18.8 kDa).

**DraSSB binding to ssDNA**

Whenever two macromolecules interact, the resulting complex
will have a larger mass than any of the single components and,
thus, it will sediment faster than the single species. Binding
SSB proteins to polymeric ssDNA [poly(dT)] results in a
dramatic increase in sedimentation rate. At 0.3 M NaCl,
20 mM KPi, poly(dT) of ~1400 nt in length has a sedimenta-
tion coefficient of 8S. Addition of different amounts of
DraSSB (3.6S) to this poly(dT) leads to an increase of this
sedimentation coefficient up to 26S at saturation. Saturation
point is reached at a stoichiometry of 50 ± 5 nt per DraSSB
dimer corresponding to a complex of ~28 SSB proteins on
each poly(dT) strand (data not shown).

All homotetrameric SSB proteins studied so far show a
dramatic decrease of tryptophan fluorescence when binding
to ssDNA (25,31,32). Figure 4 shows titrations of DraSSB
with poly(dT) at high salt (0.3 M NaCl and 20 mM KPi) and
low salt (1 mM NaCl and 1 mM KPi) conditions. For com-
parison, similar titrations of EcoSSB are shown. Under high
salt conditions, binding isotherms give a stoichiometry of
54 ± 2 nt/DraSSB(dimer) and tryptophan fluorescence is
quenched by 87 ± 2%. At low salt conditions, the stoichi-
ometry is reduced to 47 ± 2 nt/DraSSB(dimer) and the fluo-
rescence quench is diminished to 77 ± 2%. In both the cases,
the cooperative affinity is estimated to be in the range of
10^7–10^10 M^-1.

Stopped-flow analysis of the association of DraSSB to
poly(dT) at high salt (0.3 M NaCl and 20 mM KPi) (Figure 5)
is compatible with a binding mechanism where the initial
binding of the protein uses a short stretch of nucleotides that are bound in a bimolecular mechanism with an
association rate constant of 2.1 x 10^8 M^-1 s^-1. Subsequently,
the protein–nucleic acid complex rearranges in a monomol-
ecular reaction to reach its final binding site size (cf. Materials
and Methods). The association mechanism and rate constant
are in accordance with the other homotetrameric SSB proteins
investigated so far (10,25).

Since binding of SSB to poly(dT) is too strong to reliably
determine binding affinities by fluorescence titrations, we used
poly(rU) as a weakly binding substrate (33) to compare the
binding strength of EcoSSB and DraSSB. Fluorescence
titrations at high salt (0.2 M NaCl and 20 mM KPi) showed no
detectable difference in the binding affinity of these proteins
(data not shown).

For low salt concentrations (22 mM NaCl), it has been
reported that binding of EcoSSB to long ssDNA is highly
cooperative. At intermediate saturation, such cooperativity

**Figure 3.** Verification of cloned DraSSB as a genuine D. radiodurans protein
(A) and complementation assay (B). Samples were boiled in 1% SDS sample
buffer, proteins were separated in Tricine SDS–PAGE (40), blotted on PVDF
membrane and immunologically detected with a polyclonal rabbit anti-
DraSSB serum. (A) Cell extract from D. radiodurans (lane 1) compared with 200 ng
purified recombinant DraSSB (lane 2). (B) Lane 3, RDP268 (pSF1-DraSSB)
cells express only DraSSB; lane 4, RDP268 (pACYC184) cells express only
EcoSSB; and lane 5, purified recombinant EcoSSB (15 ng). Lane 3 and lane 4
contain equal amounts of cells.

**Figure 4.** Fluorescence titrations of DraSSB and EcoSSB with poly(dT) at 22°C and different salt conditions. Solid lines are theoretical binding isotherms for the
binding of a multidentate ligand to a linear polymer (23) with binding site size n and cooperative affinity ω · K as indicated below. (A) Circles: 0.375 μM DraSSB in
0.3 M NaCl, 20 mM KPi, 100 p.p.m. Tween-20; n = 53.9, ω · K = 1.2 x 10^7 M^-1, Q_s = 86.2%. Triangles: 0.375 μM DraSSB in 1 mM NaCl, 1 mM KPi, 100 p.p.m.
Tween-20; n = 47.5, ω · K = 5 x 10^6 M^-1, Q_s = 74.9%. (B) Circles: 0.29 μM EcoSSB in 0.3 M NaCl, 20 mM KPi, 0.1 mM EDTA, 100 p.p.m. Tween-20; n = 63.7,
ω · K = 1.5 x 10^7 M^-1, Q_s = 92%. Triangles: 0.44 μM EcoSSB in 1 mM NaCl, 1 mM KPi, 0.1 mM EDTA, 100 p.p.m. Tween-20; n = 39.5, ω · K = 9.5 x 10^6 M^-1,
Q_s = 70%. 
leads to a non-random distribution of ssDNA/SSB complexes. Gel electrophoresis of 50% saturated ssM13mp7 DNA showed two different species that were attributed to fully saturated and non-saturated ssDNA (32). Such a bifurcation of SSB distribution on long ssDNA can also be seen in analytical ultracentrifugation experiments with poly(dT) and SSB. In Figure 6, the c(s) distribution (19) of a poly(dT)/DraSSB mixture at low salt conditions (5 mM NaCl, 5 mM KP i, and 0.87 M glycerol) is shown. Sedimenting the mixture shortly (1 h) after preparation leads to two different complexes with ~7S and 13S, respectively. Glycerol had to be added to avoid protein aggregation and owing to its viscosity increment free poly(dT) and DraSSB sediment with 4S and 3S, respectively, in this buffer. The non-random distribution of DraSSB molecules on the poly(dT) strands is a consequence of the highly cooperative binding. If, however, the mixture of DraSSB and poly(dT) is incubated for longer periods (24–96 h), the two species merge and after 4 days incubation at room temperature only a single species sedimenting with an intermediate sedimentation constant (10S) can be detected (Figure 6). Therefore, the DraSSB molecules that initially bind in a non-random distribution redistribute very slowly on the poly(dT) strands. This slow rearrangement reflects a very large lifetime of DraSSB bound cooperatively to poly(dT). For EcoSSB under the same buffer conditions, we could also observe non-random distribution on poly(dT) by analytical ultracentrifugation confirming gel electrophoretic experiments reported previously (32). However, redistribution of EcoSSB is much slower and even after prolonged incubation (2 weeks), we could not reach fully random distribution (data not shown). Thus, cooperatively bound DraSSB more easily redistributes on poly(dT) than EcoSSB.

At high salt conditions (0.3 M NaCl and 20 mM KP i), both SSB proteins showed a random distribution on the poly(dT) strands independent of incubation time (data not shown). Therefore high DNA-binding cooperativity under high salt conditions could be excluded.

As a thermodynamic consequence of SSB proteins binding specifically to ssDNA and not to dsDNA, a destabilization of DNA double strands in the presence of SSB must be expected. Figure 7 shows that the melting temperature of poly(dA–dT) is decreased from 59 to 42°C by excess amounts of DraSSB. Under the same conditions, EcoSSB decreases the melting temperature from 59 to 37°C, indicating a weaker affinity of DraSSB for ssDNA under the conditions of the melting experiment.

**Protein–protein interactions of DraSSB**

As described earlier, the C-terminal region of EcoSSB is responsible for protein–protein interactions (28–30). Since the corresponding sequences of DraSSB and EcoSSB are highly homologous, there is a high possibility of corresponding interactions in *D. radiodurans* (9). One of the proteins functionally interacting with the C-terminus of EcoSSB is
the χ subunit of E. coli DNA polymerase III, for which qualitative and quantitative studies of the interaction have been reported previously (29,30). Thus, and since we have shown that DraSSB can take over EcoSSB function in E. coli, we decided to test protein–protein interactions of DraSSB using χ protein of E. coli as a ligand. Figure 8 shows a c(s) distribution (19) of the sedimentation of DraSSB in the presence and absence of χ. Binding of χ leads to an increase of the sedimentation coefficient from 3.6S for free DraSSB to ~4S. A binding isotherm constructed from an analysis of boundary heights shows binding of 2 χ proteins to a DraSSB dimer with an approximate affinity of $10^5$ M$^{-1}$ (Figure 8, inset). Therefore, in DraSSB all C-terminal regions are accessible for χ binding.

**DISCUSSION**

Sequence similarity classifies the ssDNA-binding protein of D. radiodurans to belong to the class of homotetrameric SSB proteins. However, the protomer of DraSSB is nearly twice the size of typical homotetrameric SSB protein subunits. Whereas the protomers of the tetrameric SSB proteins contain only one DNA-binding domain (OB-fold), the SSB protein from D. radiodurans and from other species of the *Thermus* group contain two OB-folds (7), indicating a gene duplication in the evolution of the *Thermus* group of bacteria.

From D. radiodurans cells (strain DSM20539), we amplified the ssb gene by PCR. Analysis of the DNA sequence resulted in an ORF coding for 301 amino acids and revealed sequencing errors in the genomic sequence originally published (34). These errors were recently reported by others also (9). After cloning of the DraSSB gene, we expressed the protein in E. coli and purified it to homogeneity. By western blot analysis, we could show that recombinant DraSSB protein cross-reacts with anti-EcoSSB antibodies and we identified the same protein in a total protein extract of *D. radiodurans*.

Sedimentation equilibrium experiments of DraSSB gave a molar mass of 62 kg/mol, clearly showing that DraSSB forms dimers in solution. This is in excellent agreement with the results of (9). Sedimentation velocity analysis revealed a frictional ratio of 1.5, which indicates that the DraSSB dimer must be either strongly asymmetric or globular with protrusions. DraSSB, like other proteins from the class of homotetrameric SSB proteins, contains in its C-terminal part a proline- and glycine-rich region, which is expected to be very flexible. Therefore, it seems probable that the high frictional ratio of DraSSB, as well as of the other bacterial SSB proteins (11), is caused by the extension of this region into the solution. Similarly to the other homologs, in DraSSB the proline- and glycine-rich region is succeeded by the last 10 amino acids, which are highly conserved in this class. For EcoSSB, it could be shown that this region is responsible for the interaction with other proteins involved in DNA metabolism (28–30,35). It has been speculated that the glycine- and proline-rich region acts as a spacer between the DNA-binding domain and the negatively charged region of the last 10 amino acids (36). Thus, the formation of complexes between SSB proteins and their interaction partners could be facilitated by an easy access of this region. We showed that a DraSSB dimer interacts with up to two molecules of E. coli DNA polymerase III χ subunit. Having four protomers EcoSSB protein binds up to four χ molecules (30). Thus, both C-terminal regions of the dimeric DraSSB are accessible for the interaction with other proteins. The affinity of the DraSSB/χ interaction is ~$10^5$ M$^{-1}$, similar to the affinity of the EcoSSB/χ interaction (30), despite the fact that E. coli DNA polymerase III χ subunit is not a natural interaction partner of *DraSSB* in vivo. This again demonstrates that interactions of the bacterial SSB proteins with other proteins are evolutionarily conserved and that the conserved region of the last 10 amino acids plays a crucial role in these interactions (36).

It remains to be noted that in silico analysis of D. radiodurans genome failed to reveal an ORF coding for a protein homologous to the χ subunit of DNA polymerase III from *E. coli*. Other proteins known to interact with bacterial SSB proteins [uracil DNA glycosylase (35) and primase (37)], however, have been identified in silico (Q9RWH9, Q9RWR5, respectively). Thus, it seems likely that evolutionary divergence led to a χ protein in *D. radiodurans* that could not be detected by the present in silico methods.

EcoSSB is essential for the survival of the *E. coli* cell (15), and the conserved region of the last 10 amino acids is necessary for *EcoSSB* functioning in vivo (36). Using an ssb deletion strain, we could show that DraSSB can take over the function of EcoSSB in vivo. Since DraSSB as a dimer contains only two instead of four C-termini, two C-termini seem to suffice for the in vivo function of SSB. It has been speculated that the loss of two of the four C-termini in DraSSB may lead to functional differences (9). Our data clearly show that such functional differences, even if they exist, do not play an important role in the function of SSB in vivo.

The tetrameric human mitochondrial SSB (HsmtSSB) protein cannot complement an *EcoSSB* deletion mutant strain (36), although it is structurally strongly related and shares 32%
sequence homology with EcoSSB (4,5). Since the mitochondrial SSB proteins contain no part homologous to the C-terminal region of the bacterial SSB proteins (25), this finding was not unexpected. A chimeric protein composed of the DNA-binding domain of HsmtSSB and the C-terminal third of EcoSSB is not able to take over EcoSSB function in vivo either (36). Therefore, the functional homology of the DNA-binding domains of the tetrameric EcoSSB and the dimeric DraSSB must be larger than the functional homology between the homotetrameric proteins EcoSSB and HsmtSSB.

We showed that the binding of DraSSB to ssDNA results in a quench of tryptophan fluorescence by >75% under all salt conditions used. Therefore, we used fluorescence titrations for a detailed characterization of the DNA-binding properties of DraSSB. Independent of salt concentration, the binding affinity of DraSSB and poly(dT) is larger than $10^7$ M$^{-1}$. The binding site size of DraSSB is slightly salt dependent and varies between 54 nt per DraSSB dimer at high and 47 nt at low salt concentrations (0.3 M NaCl and 20 mM KPi, 1 mM NaCl and 1 mM KPi, respectively). For EcoSSB, at least two distinctly different DNA-binding modes have been described (38). Whereas under high salt conditions, 65 nt bind per EcoSSB tetramer with almost 90% fluorescence quench, under low salt conditions 35 nt are sufficient to saturate the protein and quench its fluorescence by only 53%. Therefore, comparison of the DNA-binding properties of DraSSB and EcoSSB gives a slightly reduced binding site size for DraSSB under high salt conditions. The distinctly different binding mode of EcoSSB under low salt conditions could not be observed for DraSSB. Based on the structure of a complex of EcoSSB and two molecules of (dC)$_3$S, a model of the low salt binding mode of EcoSSB has been postulated (39). In this model, the Trp54 residues of only two subunits of EcoSSB are involved in DNA binding. The most important difference between the structures of DraSSB and EcoSSB lies in the fact that DraSSB contains two instead of one DNA-binding domain per monomer. These two binding domains (OB-folds) could evolve separately and amino acids known to be involved in DNA binding were conserved more in the C-terminal OB-fold than in the N-terminal one (7). Therefore, it has been speculated that an asymmetric DNA-binding mode, comparable with the EcoSSB low salt mode, is conserved in DraSSB in which only the C-terminal domains are used for DNA binding (7). Our present data do not support such a speculation since they show that under none of the conditions used a distinct binding mode corresponding to the low salt mode of EcoSSB could be found. The slight reduction of binding site size at high salt compared with EcoSSB is most probably owing to the somewhat smaller overall size of DraSSB. The absence of a low salt binding mode and the fact that DraSSB is functional in E.coli in vivo leads us to speculate that the low salt binding mode does not play a vital role in the life of E.coli.

Stopped-flow analysis of binding of DraSSB to poly(dT) showed that the association mechanism and rate constant are quite similar to those of the class of the homotetrameric SSB proteins (10,25).

By binding specifically to ssDNA SSB proteins destabilize DNA double strands. Comparison of DNA melting of poly(dA–dT) in the presence of DraSSB and EcoSSB respectively, revealed a weaker destabilization of the double strand by DraSSB than by EcoSSB. Since in this type of experiment the temperature is varied, differences in ssDNA-binding affinity may also reflect different temperature dependence of protein stability or DNA-binding affinity. Both GuHCl denaturation experiments in sedimentation equilibrium and irreversible denaturation of DraSSB at temperatures above 50°C in our DNA melting experiments led to the conclusion that DraSSB is less stable than EcoSSB. Therefore, we decided to measure the binding affinity at room temperature using poly(rU) as a relatively weak binding substrate. In these experiments, no difference in nucleic acid binding affinity of DraSSB and EcoSSB could be detected.

Sedimentation velocity experiments under low salt conditions showed that DraSSB bound to excess poly(dT) shows a highly asymmetric distribution caused by a large cooperativity of binding. These cooperative complexes are not at equilibrium and redistribute after 96 h of incubation. Such a formation of transiently highly cooperative complexes has also been described for EcoSSB (32). However, the redistribution of the non-randomly bound SSB proteins is much faster in the case of DraSSB.

In summary, our observations let DraSSB seem to be a normal representative of bacterial SSB proteins. Except for the absence of a low salt binding mode, DraSSB has all the essential properties of the prototype EcoSSB, including the ability to take over the vital role of EcoSSB in E.coli cells. The question as to why evolution chose to create homodimeric bacterial SSB proteins remains open.

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