Identification of amino acids stabilizing the tetramerization of the single stranded DNA binding protein from *Escherichia coli*

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Abstract Mutating the histidine at position 55 present at the subunit interface of the tetrameric *E. coli* single stranded DNA binding (SSB) protein to tyrosine or lysine leads to cells which are UV- and temperature-sensitive. The defects of both *ssb*H55Y (*ssb-1*) and *ssb*H55K can be overcome by increasing protein concentration, with the *ssb*H55K mutation producing a less stable, readily dissociating protein whose more severe replication and repair phenotypes were less easily ameliorated by protein amplification. In this study we selected and analyzed *E. coli* strains where the temperature sensitivity caused by the *ssb*H55K mutation was suppressed by spontaneous mutations that changed the glutamine at position 76 or 110 to leucine. Using guanidinium chloride denaturation monitored by sedimentation diffusion equilibrium experiments in the analytical ultracentrifuge, we demonstrate that the double mutant SSBH55KQ76L and SSBH55KQ110L proteins form more stable homotetramers as compared to the SSBH55K single mutant protein although they are less stable than wild-type SSB. Additionally, the single mutant proteins SSBQ76L and SSBQ110L form tetramers which are more resistant to guanidinium denaturation than wild-type SSB protein.

Key words: Single-stranded DNA binding protein; Protein oligomerization; Mutagenesis; Protein folding; Second-site revertant

1. Introduction

Single stranded DNA binding (SSB) proteins perform essential functions in the living cell. For many species, SSB proteins that bind to single stranded DNA (ssDNA) with little or no sequence specificity have been identified, and living cells lacking such proteins have yet to be discovered. According to their sequence or structural homology SSB proteins can be divided into several classes ranging from monomeric proteins from bacteriophages (e.g. gene-32 protein from T-even phages; [1]) to dimeric SSB proteins of filamentous phages [2] to heterotrimeric nuclear SSBs in eukaryotes (replication protein A; [3]) and finally to homotetrameric SSB proteins [4–6]. Being found in different kinds of bacteria as well as in eukaryotic mitochondria, the homotetrameric SSB proteins constitute the class most widespread among the species. These proteins all contain an amino terminal core of 100–130 amino acids conferring homotetramer formation and ssDNA binding. In addition, the bacterial SSB proteins contain a carboxy-terminal sequence of 50–70 amino acids rich in glycine and proline.

Structure-function relationships for SSB proteins have been investigated by analyzing the effect of various mutations of the amino acid sequence of *Eco*SSB. Chemical mutagenesis has generated *E. coli* strains with temperature sensitivity [7], and in one case the defect could genotypically be attributed to an *ssb* mutation [8]. In this mutant, *ssb-1*, a histidine at position 55 is replaced by a tyrosine (*ssb*H55Y; [9]) leading to a decrease in affinity of the protomers to form the homotetramer [10,11].

Replacing histidine 55 with lysine (*ssb*H55K) by site-directed mutagenesis led to proteins dissociating into monomers at concentrations where the *ssb-1* gene product (SSBH55Y) was still tetrameric. The protein still bound to ssDNA and electron micrographs of its complexes with poly(dT) showed the mutant protein to have the same tetrameric appearance as wild-type *Eco*SSB only when bound to ssDNA [11]. The apparently more severe dissociation of the *ssb*H55K tetramer as compared to the SSBH55Y (*ssb-1*) tetramer is consistent with the greater UV sensitivity and temperature-sensitive growth conferred by the *ssb*H55K mutation at a moderate copy level [12]. The phenotypic defects of both *ssb-1* and *ssb*H55K can be suppressed by increasing the gene copy number and thus the protein concentration in the cell [12,13]. However, *ssb-1* temperature sensitivity is already suppressed at pHSG575 copy level (5–6 copies, [14]), while reversal of the more extreme *ssb*H55K phenotype requires the higher expression level of pUC19 [12]. Additionally, the *ssb*H55K mutation was incapable of complementing an *ssb* deletion strain for viability when present at the low copy level of pMF3 [15].

In an attempt to identify additional amino acid residues involved in SSB subunit association, we isolated intragenic suppressors of *ssb*H55K temperature sensitivity. Since both the *ssb-1* (*ssb*H55Y) and *ssb*H55K mutations affect SSB multimerization, intragenic suppressors of either mutation might identify other amino acids involved in subunit interaction. The stronger deleterious effect of the H55K mutation on tetramerization which presumably is the basis for *ssb*H55K cells being more temperature-sensitive than *ssb-1* cells [12] makes this mutant a good candidate for easy selection of temperature-resistant intragenic suppressors.

2. Materials and methods

2.1. Revertant analysis

RDP317 is an *ssb* deletion strain wherein the chromosomal *ssb* gene is replaced with a kanamycin/neomycin drug resistance determinant (*aph*A) as described previously [16]. The mutant *ssb*H55K allele was expressed on the moderate copy level plasmid pRPZ153, an *ssb* derivative of pHSG575 [14]. A 10-ml Luria broth (LB) [17] culture was inoculated with 0.01 ml of a fresh 5-ml overnight culture of the Δ*ssb*

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strain RDP317 containing pRPZ153*ssb*H55K. The cells were grown to a predetermined optical density corresponding to approximately 10^8 cells per ml and diluted to 10 cells per ml using a 56/2 minimal salt solution [18]. A series of 10 overnight 5-ml LB cultures was inoculated with 0.1-ml aliquots to guarantee independent isolates [19]. If fewer than 7 out of 10 tubes grew, these independent overnight cultures were vigorously vortexed and 20-, 50-, and 100-fold dilutions used for plating. Low salt LB solid media (0.05% NaCl) and growth at 43°C was used to select for temperature-resistant suppressor mutations, since a higher salt concentration of 1.0% NaCl was shown to partially suppress *ssb*H55K temperature sensitivity [12].

To evaluate the potential temperature-resistant *ssb*H55K suppressor mutations, small-scale plasmid DNA preparations produced by alkaline lysis [20] were analyzed by agarose gel electrophoresis. Since gene amplification can suppress the temperature-sensitive growth phenotype of *ssb*H55K [12], we wanted to verify the presence of monomeric plasmid DNA in order to focus on DNA mutations rather than the effects of altered protein concentration. To evaluate whether the mutation allowing temperature-resistant growth was contained within the *ssb* gene, we tested the ability of the isolated plasmid to complement a chromosomal *ssb* deletion strain for viability. Monomeric plasmids were introduced via calcium chloride-mediated transformation [21] into the Δ*ssb* strain RDP317 containing the original tetracycline-resistant *ssb* plasmid pRPZ150, a pBR322 derivative [12]. By selecting the new plasmid with chloramphenicol during serial streaking and replica plating, the segregation of the original *ssb* plasmid could be traced by tetracycline sensitivity ('plasmid bumping'; [22]). Isolates which complemented the *ssb* deletion strain for viability, while remaining temperature resistant, were considered intragenic suppressors. Restriction digestion of small-scale plasmid DNA preparations confirmed the substitution of the original plasmid by the newly introduced *ssb* suppressor plasmids. Finally, cesium chloride-purified plasmid DNA from these backcrossed isolates was sequenced throughout the entire *ssb* gene to identify the suppressor mutations summarized in Table 1. In order to characterize the selected suppressor mutations, we determined UV survival in the presence and absence of *uvrA*-mediated nucleotide excision repair and SOS induction as measured by a β-galactosidase assay of a *recA-lacZ* fusion for each second-site mutation as described previously [12,23].

2.2. Buffers and reagents

All in vitro experiments were carried out in standard buffer containing 0.3 M NaCl, 20 mM KP_i, pH 7.4, 0.01 mM EDTA. *Eco*SSB concentrations were determined spectrophotometrically using an extinction coefficient for the tetramer of 113 000 M^{-1} cm^{-1} at 280 nm [24] and are given in units of monomers throughout the text. Poly(dT) was purchased from Pharmacia (Freiburg) and had an average length of 1400 nucleotides. Guanidinium chloride (biochemical grade) was purchased from Merck, Darmstadt.

2.3. Construction of the mutant Ecossb genes and protein purification

All mutant SSB proteins are designated according to their differences compared to wild-type SSB (e.g. H55KQ76L designating a protein with amino acids H and Q of wild-type SSB at positions 55 and 76 substituted by K and L, respectively). The construction of *ssb*H55K and purification of the protein was described earlier [11]. All other mutations were constructed in the *E. coli ssb*-gene containing plasmid pSF1 [25] either by gapped duplex mutagenesis [26] or by introducing appropriate restriction fragments from the revertants. The complete sequence of all mutant genes was confirmed by DNA sequencing. Proteins were prepared as described in [27]. All proteins were more the 95% pure as judged from SDS-PAGE [28].

Table 1
Intragenic suppressors of *ssb*H55K temperature sensitivity

Suppressors	Codon change	Frequency
*ssb*H55K and Q76L	CAG > CTG	11 (61%)
*ssb*H55K and Q110L	CAG > C**T**G	3 (17%)
*ssb*H55I	AAA > A**T**A	2 (11%)
*ssb*H55T	AAA > A**C**A	2 (11%)
		18

Cesium chloride-purified plasmid DNA was prepared from derivatives of the Δ*ssb* strain RDP317 containing backcrossed intragenic suppressor isolates of *ssb*H55K temperature sensitivity on pRPZ153. DNA sequencing analysis of the entire *ssb* gene fragment yielded the different types of suppressor mutations listed above.

2.4. Physicochemical measurements

Analytical ultracentrifugation was performed in a Beckman XL-A analytical ultracentrifuge equipped with absorption scanner optics using an AN-50 Ti 8-hole rotor. Sedimentation-diffusion-equilibrium experiments were done using short columns (approx. 3 mm) and Fluorinert FC-43 (ABCR, Karlsruhe) as artificial bottom. When the measured concentration profile remained unchanged for at least 12 h we assumed equilibrium to be attained. Apparent molecular masses were calculated by fitting the ideal concentration gradient for a single species [29] to the measured concentration profiles using the program package AKKUPROG [30]. Partial specific volumes of all proteins used were calculated from amino acid composition with no correction for the influence of guanidinium chloride.

In sedimentation velocity experiments the apparent sedimentation coefficient $s^{app}_{20,W}$ for the sedimenting boundary was evaluated with the program package AKKUPROG [30] fitting the time dependent concentration profiles calculated with Lamm's differential equation [31] for a single sedimenting species to the measured data.

Stopped-flow kinetics were measured in a modified version of a Durrum-Gibson stopped-flow apparatus as described previously [24]. The measurements were carried out in standard buffer containing 500 ppm Tween 20.

Prior to velocity centrifugation or stopped flow analysis proteins diluted from stock solutions were incubated at least 16 h at room temperature to ensure dissociation equilibrium.

3. Results

3.1. Revertants

The substitution of glutamine by leucine at positions 76 and 110 allowed intragenic second-site suppression of *ssb*H55K temperature sensitivity. Although the most abundant suppressor mutation was *ssb*Q76L (61%; Table 1), less frequent intragenic suppressors included the second-site *ssb*Q110L mutation and the same-site mutations at histidine 55 (17% and 11%; Table 1). The same-site mutations *ssb*H55I and *ssb*H55T may have grossly restored wild-type properties of SSB as suggested by previous histidine substitutions by isoleucine and phenylalanine which demonstrated no measurable change in protein properties [11]. The *ssb*H55I and *ssb*H55T mutations were not examined further in this study.

The frequency at which the two different second-site mutations were isolated seems to correlate with their ability to suppress *ssb*H55K ultraviolet (UV) sensitivity. While the second-site mutation leading to *ssb*H55KQ76L fully reverses the 100-fold reduction from wild-type survival at 54 J/m^2 seen with *ssb*H55K alone (Fig. 1), the *ssb*H55KQ110L mutation

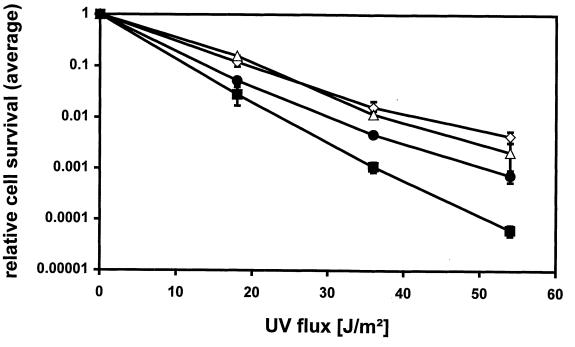

Fig. 1. UV sensitivity of derivatives of Δ*ssb* strain RDP317 containing wild-type *ssb* (◇), *ssb*H55K (■), *ssb*H55KQ76L (△), and *ssb*H55KQ110L (●/◒) on pRPZ153. Irradiation was done at an UV flux of 0.6 J m^{-2} s^{-1}. The UV survival curve for *ssb*H55KQ76L is indistinguishable from that of wild-type *ssb*.

only partially suppresses UV sensitivity. It should also be noted that the *ssb*Q76L single mutation produces UV survival equivalent to that of *ssb*$^+$ (data not shown). Surprisingly, *ssb*H55K shows greater UV survival than *ssb*$^+$ in a Δ*uvrA* background [23]; however, both *ssb*H55KQ76L and *ssb*H55KQ110L suppressor mutations possess UV survival comparable to wild-type *ssb* in the absence of *uvrA*-mediated repair (data not shown). Since *ssb*H55K and *ssb*$^+$ possess identical UV survival in a non-inducible *lexA3* background, we assume the greater UV resistance conferred by *ssb*H55K in a Δ*uvrA* strain reflects a greater amount of SOS induction. This idea is supported by the extent to which SOS is induced as measured with a *recA-lacZ* fusion with a β-galactosidase assay; *ssb*H55K, *ssb*H55KQ76L, and *ssb*H55KQ110L have slightly greater induction than wild-type *ssb* while the *ssb*Q76L mutant was indistinguishable from the *ssb*$^+$ control (Table 2).

3.2. Protein oligomerization

To probe for the structure function relationship of the revertant SSB proteins, we inserted genes for the revertant double mutants *ssb*H55KQ76L and *ssb*H55KQ110L and the single mutants *ssb*Q110L and *ssb*Q76L into the overproducing vector pSF1 [25]. The tendency of position 55 mutant SSB proteins to dissociate can be observed by measuring the concentration dependence of the apparent sedimentation coefficient. In such an experiment dissociation of the homotetramer at low protein concentrations leads to a reduction of the apparent sedimentation rate constant $s^{app}_{20,W}$. Fig. 2 shows the dependence of $s^{app}_{20,W}$ for SSBH55K, SSBQ76L, SSBQ110L and combinations thereof. At high concentrations all proteins form homotetramers sedimenting with approx. 4 S. The slightly higher sedimentation coefficient of SSBH55KQ110L reflects the tendency of this protein to form aggregates larger than tetramers (see below). While SSBH55K readily dissociates at low concentrations, this dissociation is shifted to much lower concentration by the suppressor mutations SSBH55KQ76L and SSBH55KQ110L and only partial dissociation can be observed. For the single mutants SSBQ76L and SSBQ110L, no dissociation can be detected.

Another test for tetramer stability is the resistance of the tetramer against guanidinium chloride-induced denaturation. Such denaturation leads to monomeric proteins and the transition can be observed by measuring the apparent molecular mass from sedimentation-diffusion equilibrium experiments in the analytical ultracentrifuge. Fig. 3 shows the dependence of

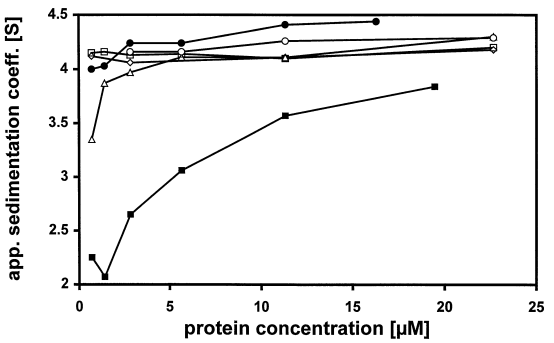

Fig. 2. Concentration dependence of the apparent sedimentation coefficients $s^{app}_{20,W}$ of wild-type SSB (◇), SSBH55K (■), SSBH55KQ76L (△), SSBH55KQ110L (●), SSBQ76L (○), SSB Q110L (□). Lines to guide the eye.

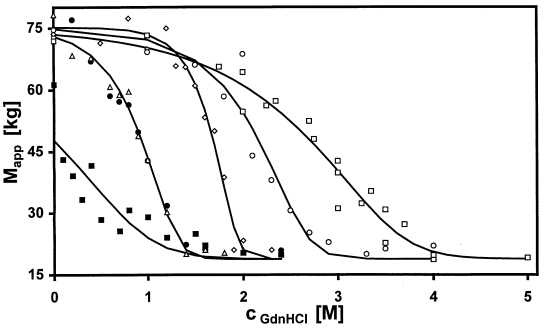

Fig. 3. Guanidinium denaturation of (8 μM) mutant *Eco*SSB proteins as observed by sedimentation-diffusion equilibrium in the analytical ultracentrifuge. Apparent molecular masses are plotted for wild-type SSB (◇), SSBH55K (■), SSBH55KQ76L (△), SSBH55KQ110L (●), SSBQ76L (○), SSB Q110L (□). Lines to guide the eye. SSBH55KQ110L in absence of guanidinium chloride showed an apparent molecular mass of 92 kDa.

the apparent molecular mass on guanidinium chloride concentration. All mutant and wild-type *Eco*SSB proteins could be dissociated into monomers. The resistance against guanidinium chloride increases in the following order:
SSBH55K < SSBH55KQ110L ≅ SSBH55KQ76L < wild-type < SSBQ110L ≅ SSBQ76L. Again, the tendency of SSBH55KQ110L to form aggregates is reflected by the large apparent molecular mass in the absence of guanidinium chloride.

3.3. Binding to single stranded DNA

For SSBH55K we have shown that this protein forms complexes with poly(dT) very similar to wild-type SSB. However, the kinetics of complex formation differ drastically: while wild-type *Eco*SSB reacts with poly(dT) at an almost diffusion controlled rate (2.5×10^8 M^{-1} s^{-1}, [24]) SSBH55K needs several steps ranging from 100 ms to several hours to completely react with poly(dT) [11]. This slow reaction was interpreted as a slow refolding and association of the dissociated protein that had to occur to enable DNA binding. Thus, since tetramerization equilibrium is influenced by ssDNA binding in a slow reaction, fast kinetic measurements can be used to probe for tetrameric SSB active in DNA binding. In a stopped flow experiment both revertants SSBH55KQ76L and SSBH55KQ110L clearly show biphasic association kinetics (Fig. 4). At high concentration this biphasic behavior disappears and the proteins react with poly(dT) with an association rate constant of 3.3×10^8 M^{-1} s^{-1} which is very similar to that found for wild-type SSB.

Table 2
Support of SOS induction

ssb allele	Relative SOS induction level
ssb$^+$	3.50 ± 0.27
*ssb*H55K	4.53 ± 0.18
*ssb*H55KQ76L	4.52 ± 0.24
*ssb*H55KQ110L	4.25 ± 0.21
*ssb*Q76L	3.58 ± 0.17

Derivatives of Δ*uvrA* strain RDP320 containing the λ(*recA-lacZ*) fusion and the *ssb* mutations present on pHSG575 were analyzed by a minimum of three β-galactosidase assays as described previously [23]. The amount of induction was determined by a ratio of the induced to non-induced levels of enzyme units produced per milliliter [17] and is shown with one standard deviation.

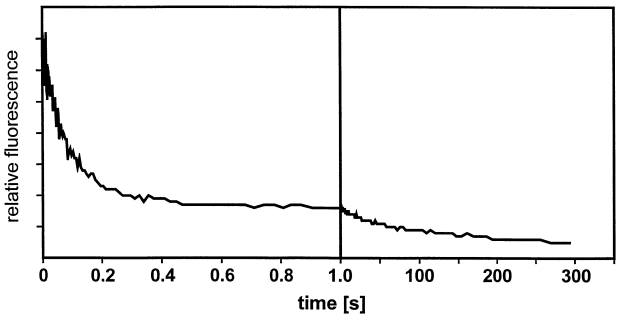

Fig. 4. Association kinetics for the reaction of 250 nM SSBH55KQ110L with 4.1 μM nucleotides of poly(dT) as observed by fluorescence detected stopped flow. A fast process representing the association of intact tetrameric protein (app. 85% of total) to poly(dT) is followed by a slow reaction which can be interpreted as slow tetramerization and binding.

4. Discussion

We have selected and analyzed *E. coli* strains where the temperature sensitivity caused by the *ssb*H55K mutation was suppressed by spontaneous mutations that changed the glutamine at position 76 or 110 to leucine. We have found that the double mutant SSBH55KQ76L and SSBH55KQ110L proteins form more stable homotetramers as compared to the SSBH55K single mutant protein although they are less stable than wild-type SSB. Both of these glutamine to leucine mutations isolated as suppressors of temperature sensitive growth also suppressed, albeit to differing degrees, the UV sensitivity phenotype associated with *ssb*H55K mutation. Additionally, the single mutant proteins SSBQ76L and SSBQ110L form tetramers which are more resistant to guanidinium denaturation than wild-type SSB protein. The fact that amino acids at these positions are involved in tetramer formation has helped in arranging the subunits in correct tetramers within the crystal structure of the aminoterminal core of *Eco*SSB [4].

For the class of homotetrameric SSB proteins, the ability to form the homotetramers seems to be an essential requirement for a functional protein. An increased tendency to dissociate leads to temperature and UV sensitivity of such mutant strains [12]. Substitutions of histidine at position 55 of *Eco*SSB can lead to such an increased tendency to dissociate [11]. The histidine at position 55 appears to participate in interactions between two subunits by hydrogen bonding to various residues of the opposing monomer [5]. Thus a change to a charged or more bulky side chain like lysine or tyrosine destroys these spatial interactions. A hydrophobic interaction introduced at position 76 or 110 by exchanging a glutamine to a leucine then stabilizes the tetramer again. It is interesting to note that homologous SSB proteins not containing the highly conserved histidine residue at position 55, such as the *Saccharomyces cerevisiae* mitochondrial SSB [32] and SSB encoded on the *Klebsiella aerogenes* RK2 plasmid [33] and by *Bacillus subtilis* [34], possess a leucine at position 76 reflecting the most commonly isolated intragenic suppressor of *ssb*H55K temperature sensitivity. For the H55K suppressors *ssb*H55KQ76L and *ssb*H55KQ110L the improved protein stability alone may alter UV survival to values obtained with wild-type SSB. When comparing both second-site suppressors the stability of the SSBH55KQ76L mutant tetramer is similar or even less than that of SSBH55KQ110L. However, this does not correlate with the greater UV resistance of *ssb*H55KQ76L and suggests that the glutamine residue at position 110 plays some other role in the in vivo function of SSB than in tetramerization.

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