Altered Tethering of the SspB Adaptor to the ClpXP Protease Causes Changes in Substrate Delivery*

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SspB is a dimeric adaptor protein that increases the rate at which ssrA-tagged substrates are degraded by tethering them to the ClpXP protease. Each SspB subunit consists of a folded domain that forms the dimer interface and a flexible C-terminal tail. Ternary delivery complexes are stabilized by three sets of tethering interactions. The C-terminal XB peptide of each SspB subunit binds ClpX, the body of SspB binds one part of the ssrA-tag sequence, and ClpX binds another part of the tag. To test the functional importance of these tethering interactions, we engineered monomeric SspB variants and dimeric variants with different length linkers between the SspB body and the XB peptide and employed substrates with degradation tags that bind ClpX weakly and/or contain extensions between the binding sites for SspB and ClpX. We find that monomeric SspB variants can enhance ClpXP degradation of a subset of substrates, that doubling the number of tethering interactions stimulates degradation via changes in $K_m$ and $V_{max}$, and that major alterations in the length of the 48-residue SspB linker cause only small changes in the efficiency of substrate delivery. These results indicate that the properties of the degradation tag and the number of SspB-ClpX tethering interactions are the major factors that determine the extent to which the substrate and ClpX are engaged in ternary delivery complexes.

Adaptor proteins frequently regulate the substrate specificity of AAA$^+$ enzymes, which function as ATP-powered molecular machines in a wide assortment of cellular processes (1). For AAA$^+$ proteases, adaptors can alter substrate choice by enhancing or inhibiting the degradation of specific proteins (2–4). Determining the detailed molecular mechanisms by which different proteolytic adaptors function is important both for a deeper understanding of the determinants of degradation specificity and for the rational design of synthetic adaptors.

Escherichia coli ClpXP is an ATP-dependent protease (3). Its biological roles include degrading ssrA-tagged proteins and an N-terminal fragment of RseA that inhibits the o$^o$ transcription factor (7, 9). Flexible tails allow tethering of the SspB dimer to ClpX via interactions in which XB peptide motifs at the extreme C terminus of each SspB subunit dock with N-domains on the ClpX ring (Refs. 10 and 11; see Fig. 1A). The substrate binding domain of each SspB subunit contains a groove that serves as a docking site for part of the ssrA-tag sequence or a peptide sequence in RseA (12, 13). Thus, binary interactions link the substrate to SspB and this adaptor to ClpX. The ternary complex is further stabilized by interactions between the substrate degradation tag and the central pore of the ClpX ring (14, 15). Substrate delivery can be blocked by mutations that prevent binding of the substrate to SspB or binding of SspB to ClpX, demonstrating that the ability of SspB to tether substrates to ClpX is critical for its function as an adaptor (9, 10).

The ssrA tag (AANDENYALAA in E. coli) is a peptidic sequence that is added to the C terminus of nascent proteins when ribosomes stall during translation (3–5). The AANDENY portion of the tag contains binding determinants for SspB, whereas the LAA sequence is recognized by ClpX (Ref. 16; Fig. 1C). Proteins containing an ssrA tag are recognized and degraded by ClpXP alone, but degradation at low substrate concentrations occurs more rapidly in the presence of SspB (9, 10). Recent studies establish that proteins with modified ssrA tags ending in the sequence DAS instead of LAA are poor substrates for ClpXP unless SspB is present (17). In delivery complexes, ClpX and SspB can simultaneously contact the ssrA tag of a protein substrate (Ref. 14; Fig. 1A), but their resulting close proximity appears to cause a clash that reduces complex stability (18). Indeed, SspB lacking its tethering tails inhibits degradation, and extended tags containing extra residues between
Substrate Delivery by Variant SspB Adaptors

the sites that bind SspB and ClpX improve adaptor-mediated substrate degradation (10, 18).

Crystallographic studies reveal how SspB binds to the degradation tags of substrates and how the XB motif of SspB binds to the N-domain of ClpX (12, 13, 19). Although, these static interactions are critical for substrate delivery, dynamic interactions mediated by unstructured portions of SspB play an equally important role. For example, both flexible C-terminal tails of an SspB dimer normally tether the adaptor to ClpX. This bivalent interaction is functionally important, as a dimer with one full-length subunit and one subunit missing its C-terminal tail binds ClpX weakly and fails to enhance ClpXP degradation of ssrA-tagged proteins (11). Monovalent tethering of SspB to ClpX via single tail may result in binding that is too weak to overcome the proposed clash during substrate delivery, but other explanations for the importance of bivalency are also possible. For example, SspB variants with just one C-terminal tail might dissociate too quickly from ClpX to allow substrate delivery, or monovalent tethering might be required for proper orientation of the substrate for handoff to the enzyme. In SspB orthologs, the unstructured linkers that connect the substrate binding domain to the XB motif vary in length from 20 to 55 residues and show little sequence conservation. Although this distribution of natural linker lengths suggests significant tolerance, it is unclear if very short SspB linkers would still permit effective bivalent tethering or if longer linkers would keep the substrate and ClpX sufficiently close to allow efficient delivery.

Here, we use mutant SspB adaptors and substrates bearing ssrA-like degradation tags with different properties to explore the importance of the number and type of tethering interactions in adaptor-mediated substrate delivery to ClpXP. We find that monomeric variants of SspB can enhance ClpXP degradation but only for substrates bearing extended degradation tags and with reduced efficiency compared with dimeric SspB. The efficiency of substrate delivery by dimeric SspB varies only modestly as a function of linker length, and SspB variants with almost no linker or with double-length linkers still function as efficient adaptors. The implications of these findings for the mechanism of substrate delivery by SspB and for general models of adaptor function are discussed.

EXPERIMENTAL PROCEDURES

Construction of Mutant Variants—Variants of the ssrA tag and SspB used in this study are depicted in Fig. 1. Plasmids encoding GFP bearing LAA (wild-type ssrA), LAA + 4, DAS, or DAS + 4 tags at their C terminus were generated by PCR amplification/mutagenesis of a gene encoding GFP-His6-ssrA (14) followed by ligation into pET11d. Monomeric SspB was generated by introducing eight different mutations (see below) into a plasmid-borne gene for E. coli SspB. Another monomeric SspB with an N-terminal His10 tag was constructed in pET24d for biophysical studies. The two-tailed SspB monomer was assembled in a modular fashion to contain the N-terminal sequence (MGDDRGRGPRALRVVK (the XB motif is in bold) followed by the sequence for residues 113–154 of E. coli SspB, a His6 tag, and the sequence for residues 1–165 of monomeric SspB. To facilitate construction, an Ndel site was present between the coding sequences for the His6 tag and monomeric SspB, and residues 112–113 of monomeric SspB were mutated from Asp-Thr to Gly-Ser to introduce a BamHI site. Individual DNA fragments were amplified by PCR and digested with the appropriate restriction enzymes, and the final construct was ligated between the NcoI and XhoI sites of pET24d. The N-tailed SspB dimer was generated by digesting the pET24d plasmid encoding the two-tailed SspB monomer with Ndel and BamHI and inserting an Ndel-BamHI fragment encoding residues 1–112 of dimeric SspB followed by a stop codon.

SspB tail-length variants were initially made as part of another study and were constructed in a background consisting of residues 1–106 of Hemophilus influenzae SspB Y44C followed by residues 108–165 of E. coli SspB (14). The substrate binding domains of H. influenzae and E. coli SspB have similar sequences and the same three-dimensional fold (12, 13). Moreover, a hybrid consisting of the H. influenzae substrate binding domain and the unstructured E. coli tail is fully active (11). The Y44C mutation allows cross-linking of ssrA peptides to SspB but does not alter adaptor activity significantly (14). Importantly, all activities of tail-length variants were compared with those of the otherwise isogenic parental mole (designated SspB21,48, where 48 is the length of the unstructured linker region). Tail-length variants were generated by mutagenesis of the pET21b derivative encoding SspB21,48. Variants with shorter tails were generated by PCR amplification of the SspB1,48 plasmid with phosphorylated primers designed to delete the codons for residues 134–155 (SspB1,45) or residues 113–155 (SspB1,15) of the E. coli tail followed by ligation to generate a variant with the longer tail of SspB1,91, a SacII site was introduced by silent mutation of the codons for residues 153 and 154 in the parent plasmid, the resulting plasmid was digested with SacII and HindIII, and a SacII-HindIII PCR fragment encoding residues 113–165 of the E. coli SspB tail was inserted by ligation.

Plasmids encoding E. coli ClpX, E. coli ClpP-His6, and a His6-tagged variant of chimeric SspB with the H. influenzae substrate binding domain and the E. coli tail have been described (11, 20, 21). Plasmids encoding E. coli SspB and His6-tagged E. coli SspB were similar to those encoding the chimeric variants except for the source of the coding sequences.

Protein Production—All GFP variants and untagged monomeric SspB were expressed in E. coli BB101 cells, a derivative of strain X90 (f’ lac pro ara △(lac-pro) naI A argE (am) rif’ thi-1) harboring the slyD:kan mutation and the Ade3 prophage (22). Radiolabeled GFP-DAS and GFP-DAS+4 were purified after growth in media containing [35S]Met as described (5). The N-tailed SspB dimer and the two-tailed SspB monomer were expressed in E. coli strain X90 (Ade3). His6- monomeric SspB was expressed in E. coli BLR cells (Novagen). For purification of all dimeric SspB variants except the N-tailed dimer, untagged proteins were coexpressed in BLR cells with a His6-tagged dimerization partner (E. coli His6-SspB for E. coli SspB variants; the H. influenzae E. coli His6-SspB hybrid for linker-length variants) to facilitate purification. Heterodimers containing tagged and untagged subunits were purified by Ni2+ - nitritoltriacetic acid affinity, and the untagged proteins were

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5 The abbreviations used are: GFP, green fluorescent protein; ATPγS, adenosine 5’-O-(thiotriphosphate).
eluted under denaturing conditions and refolded (14). GFP variants, N-tailed SspB dimer, two-tailed SspB monomer, and His<sub>10</sub>-monomeric-SspB were purified by Ni<sup>2+</sup>-nitrilotriacetic acid affinity. Untagged monomeric SspB was purified using a protocol similar to that described for wild-type SspB (9) and was a gift from G. Hersch (MIT). ClpX was expressed and purified as described (23). ClpP-His<sub>6</sub> was expressed and purified as described (20) and was a gift from Andreas Martin (MIT).

**Computational Design and Biophysical Characterization of Monomeric SspB**—Calculations based on the structure of the *H. influenzae* SspB dimer (Ref. 12; PDB code 1OU9) showed that positions 5, 8, 11, 12, 15, 16, 19, 22, 101 changed classification as core, surface, or boundary residues in the absence of the partner subunit (24). In the design, these dimer-interface positions were restricted to Asp, Asn, Glu, Gln, His, Lys, Ser, Thr, and Arg. Basic residues at position 8 and acidic residues at positions 19 and 22 were excluded to prevent unfavorable helical-dipole interactions (25). The ORBIT protein design program was used to optimize amino acid identity and geometry using an energy function based on the DREIDING force field that included a van der Waal's potential (atomic radii scaled by 0.9), a coulombic function based on the DREIDING force field that included a van der Waal's potential (atomic radii scaled by 0.9), a geometric hydrogen-bond potential with a dielectric constant of 78, and a statistical term to account for β propensity at position 101 (24, 26–28). The final design left Thr-101 unchanged and included eight mutations (L6R, R9E, Y12Q, L13K, A16E, F17K, W20E, D23K), which were introduced into the *E. coli* SspB coding sequence as described above.

For denaturation studies, 3 μM His<sub>10</sub>-monomeric-SspB (in 50 mM potassium phosphate, pH 7.6) was incubated with different concentrations of guanidine-HCl at 25 °C for 1 h or more before measuring the circular dichroism ellipticity at 222 nm. Analytical ultracentrifugation (Beckman XLA instrument; Ti-60 rotor; 20 °C; 16,000 rpm; 24 h equilibration) was performed using 80 μM His<sub>10</sub>-monomeric-SspB in 50 mM potassium phosphate, pH 7.5, 150 mM potassium chloride, and 0.5 mM dithiothreitol. Binding of *E. coli* SspB and His<sub>10</sub>-monomeric-SspB to a fluorescein-labeled ssrA peptide was performed as described (14).

**ClpXP Degradation**—A series of increasing concentrations of GFP-ssrA and GFP-LAA<sup>+</sup>4 were degraded at 30 °C using 100 nM ClpX<sub>6</sub> and 300 nM ClpP<sub>14</sub> in PD buffer (25 mM HEPES, pH 7.6, 200 mM KCl, 5 mM MgCl<sub>2</sub>, 0.032% Nonidet P-40, and 10% glycerol) with an ATP regeneration system containing 4 mM ATP, 16 mM creatine phosphate, and 0.32 mg/ml creatine phosphokinase. When SspB or variants were present, the adaptor concentration in subunit equivalents was equal to the substrate concentration. The GFP substrate with or without SspB was incubated for 2 min at 30 °C as were ClpXP and the remaining reaction components. These mixtures were combined (60 μl total), and degradation rates were measured as a loss of GFP fluorescence. A series of increasing concentrations of <sup>35</sup>S-labeled GFP-DAS and GFP-DAS<sup>+</sup>4 was degraded in a similar manner in a 100-μl reaction volume, and initial degradation rates were determined by the release of trichloroacetic acid-soluble peptides (20). For all substrates, initial rates of degradation were plotted versus substrate concentration and fitted to the Hill equation (see “Results”).

**Competition for ClpX Binding**—A fluorescein-SspB-ssrA molecule was assembled as described (29) by cross-linking an ssrA peptide with the A2C mutation to an SspB subunit containing the Y44C mutation in a chimeric *H. influenzae-E. coli* variant that forms an obligate heterodimer (11). Binding of fluorescein-SspB-ssrA (250 nm) to ClpX (200 nm) at 30 °C was assayed in PD buffer plus 4 mM ATP/γS with varying concentrations of each SspB variant as competitors. Fluorescence anisotropy values were measured for 2 min and averaged.

**RESULTS**

**SspB and Substrate Variants**—To probe the mechanism by which SspB delivers ssrA-tagged proteins to ClpXP, we gener-
Substrate Delivery by Variant SspB Adaptors

FIGURE 2. Biophysical properties of the designed SspB monomer. A, analytical ultracentrifugation (20 °C; 16,000 rpm) of 80 μM SspB monomer after 24 h. The solid fitted line predicts a molecular weight of 20 kDa, the value expected for the monomer; the dashed line represents the expected protein distribution for a dimer. The residuals of the fit are shown. B, denaturation of 3 μM SspB monomer by guanidine-HCl. Unfolding was monitored by changes in circular-dichroism ellipticity at 222 nm. C, binding of a fluorescein-modified ssrA peptide to the E. coli SspB dimer (squares; fitted $K_D = 2.3 \mu M$) or the SspB monomer (circles; fitted $K_D = 6.6 \mu M$) assayed by fluorescence anisotropy.

Monomeric SspB had different effects on the kinetics of ClpXP degradation studies, we used GFP substrates bearing either a wild-type or mutant ssrA tag (Fig. 1C). In one variant, the C-terminal LAA of the wild-type ssrA tag was replaced with DAS. Extended tags with four residues inserted between the tag segment that binds SspB and the three C-terminal residues were also used (LAA+4 or DAS+4). DAS-tagged substrates are useful probes of SspB function because their degradation by ClpXP is almost completely SspB-dependent (17). In previous studies, substrates bearing extended tags were found to be degraded more efficiently than the corresponding shorter-tag substrates when ClpXP and SspB were present (17, 18). To determine kinetic parameters, rates of ClpXP degradation were determined at a series of substrate concentrations. For some substrates and SspB variants, the change in the degradation rate as a function of concentration showed positive cooperativity, suggesting that ternary adaptor-substrate-enzyme complexes assembled at concentrations where binary complexes were not significantly populated. To model this behavior, data were fit to a form of the Hill equation (rate = $V_{max}/(1 + (K_m/\text{[substrate]}^n))$, where $V_{max}$ is the maximal velocity, $K_m$ is the substrate concentration required to achieve half-maximal velocity, and $n$ is the Hill coefficient.

Monomeric SspB Variants—are fit to a form of the Hill equation (rate not significantly populated. To model this behavior, data assembled at concentrations where binary complexes were suggesting that ternary adaptor-substrate-enzyme complexes examination of concentration showed positive cooperativity, sug-

Monomeric SspB Enhances Degradation of Substrates with Extended Tags—To create a monomeric variant of E. coli SspB, we mutated hydrophobic residues that normally form the dimer interface to charged and polar residues. The purified protein sedimented as a monomer in analytical ultracentrifugation experiments (Fig. 2A), displayed cooperative guanidine-HCl denaturation (Fig. 2B), and bound to an ssrA peptide with a $K_D$ of 7 μM (Fig. 2C). Another variant contained monomeric SspB preceded by a flexible region of polypeptide with a second XB motif near the N terminus (Fig. 1B). We refer to this mutant, which contains N-terminal and C-terminal tails that allow tethering to ClpX, as the two-tailed monomer. A third mutant, called the N-tailed dimer, consisted of dimeric SspB with the N-terminal tail used in the two-tailed monomer but with no C-terminal tail (Fig. 1B).

The ability of monomeric SspB to enhance ClpXP proteolysis depended on the nature of the degradation tag present on the GFP-reporter substrate. For example, monomeric SspB caused no significant change in ClpXP degradation of GFP-ssrA but increased the rate at which GFP-DAS was degraded (Fig. 3A). Similarly, the monomeric adaptor failed to enhance degradation of GFP-DAS but stimulated degradation of GFP-DAS+4 (Fig. 3B). Thus, monomeric SspB enhanced degradation of substrates with extended tags but failed to stimulate degradation of substrates with normal-length tags.

Monomeric SspB had different effects on the kinetics of ClpXP degradation of the two extended-tag substrates. For GFP-LAA+4, a good intrinsic substrate (18), monomeric SspB caused little change in $V_{max}$ and affected degradation principally by decreasing $K_m$ severalfold (Fig. 3C; Table 1). For GFP-DAS+4, a poor intrinsic ClpXP substrate (17), monomeric SspB increased $V_{max}$ at least 2-fold and also decreased $K_m$ more than 10-fold (Fig. 3D; Table 1). In the presence of monomeric SspB, GFP-LAA+4 was degraded faster than GFP-DAS+4, but ClpXP proteolysis of the latter substrate was enhanced to a much greater extent when compared with adaptor-free reactions. Thus, monomeric SspB increases the rate at which ClpXP degrades extended-tag substrates by strengthening enzyme-substrate interactions and by increasing the maximal rate of degradation.
Next, we assayed ClpXP degradation of GFP-DAS+4 in the presence of the two-tailed SspB monomer or the wild-type SspB dimer. As shown in Fig. 3D, both adaptors were more efficient than monomeric SspB in enhancing degradation of GFP-DAS+4. Moreover, in both cases, increased degradation efficiency resulted from increases in $V_{\text{max}}$ as well as decreases in $K_m$ (Fig. 3D; Table 1), although wild-type SspB caused larger changes in both kinetic parameters than did the two-tailed monomer. Several factors could explain these differences. For example, the SspB monomer binds the ssrA degradation tag about 3-fold more weakly than the wild-type SspB dimer (Fig. 2C), suggesting that the tag binding groove is slightly distorted in the monomer. Weaker tag binding would reduce the efficiency of substrate delivery. It is also possible that the N-terminal tail of the two-tailed monomer does not interact as strongly with ClpX as the second C-terminal tail of the SspB dimer. To address the latter possibility, we assayed degradation of GFP-DAS+4 in the presence of the N-tailed SspB dimer. This dimeric adaptor was less efficient than the two-tailed monomer in delivering GFP-DAS+4 to ClpXP but was slightly more efficient than monomeric SspB in enhancing degradation (Table 1). Thus, bivalent tethering of SspB or its variants to ClpXP enhances the efficiency of substrate delivery. However, the two C-terminal tails of the wild-type SspB dimer provide better tethering than the N-terminal and C-terminal tails of the two-tailed monomer, which in turn are superior to two N-terminal tails attached to an SspB dimer.

**Substrate Delivery by Variant SspB Adaptors**

To probe the importance of tail length in substrate delivery, we constructed mutants with link-
ers of 5 residues (SspB\textsuperscript{L1.5}), 25 residues (SspB\textsuperscript{L1.25}), 48 residues (SspB\textsuperscript{L1.48}), and 91 residues (SspB\textsuperscript{L1.91}) between the substrate binding domains of an SspB dimer and the LRVVK sequences that comprise the C-terminal XB motifs (Fig. 1B). The SspB\textsuperscript{L1.48} variant contains the substrate binding domain of \textit{H. influenzae} SspB, a 48-residue linker from \textit{E. coli} SspB (residues 113–160), and the \textit{E. coli} XB motif (residues 161–165). A related chimeric protein efficiently delivers ssrA-tagged substrates to \textit{E. coli} ClpXP (11). The variants with 5-, 25-, and 91-residue linkers were derived by deletion or duplication of the linker region of SspB\textsuperscript{L1.48}.

The rate of ClpXP degradation of 0.3 \textmu M GFP-DAS+4 was measured in the presence of 0.15 \textmu M concentrations of the SspB\textsuperscript{L1.5}, SspB\textsuperscript{L1.25}, SspB\textsuperscript{L1.48}, and SspB\textsuperscript{L1.91} dimers (Fig. 4A). Under these conditions, the fastest degradation was observed for the SspB variant with the 48-residue linker. Degradation was roughly 60\% as fast for SspB\textsuperscript{L1.25}, 30\% as fast for SspB\textsuperscript{L1.5}, and 20\% as fast for SspB\textsuperscript{L1.91}. Thus, the length of the SspB linker region affects the efficiency of substrate delivery to ClpXP. However, even the slowest adaptor-mediated reaction (SspB\textsuperscript{L1.91}) was almost 40-fold faster than ClpXP degradation of 0.3 \textmu M GFP-DAS+4 in the absence of an adaptor. Hence, changing the length of the SspB linker from just a few residues to more than 90 residues does not preclude substantial rates of substrate delivery to ClpXP, although optimal delivery was observed when the linker was the wild-type length.

\[ V_{\text{max}} \] for ClpXP degradation of GFP-DAS+4 was within error in the presence of the SspB\textsuperscript{L1.25}, SspB\textsuperscript{L1.48} and SspB\textsuperscript{L1.91} variants and was only slightly lower for SspB\textsuperscript{L1.5} (Table 2). By contrast, the apparent \( K_m \) values for these mutants varied over a 4–5-fold range, with the lowest value observed for SspB\textsuperscript{L1.48} and the highest value for SspB\textsuperscript{L1.91}. These results suggest that linker length affects the affinity of these dimeric adaptors for ClpX. To address this issue directly, we measured the ability of the SspB\textsuperscript{L1.5}, SspB\textsuperscript{L1.25}, SspB\textsuperscript{L1.48}, and SspB\textsuperscript{L1.91} proteins to compete for ClpX binding to a fluorescein-modified SspB-ssrA complex (14). In this assay, displacement of the fluorescent complex from ClpX by unlabelled SspB variants results in a decrease in fluorescence anisotropy. The SspB\textsuperscript{L1.48} protein bound ClpX most strongly in this competition assay followed by SspB\textsuperscript{L1.25}, SspB\textsuperscript{L1.91}, and SspB\textsuperscript{L1.5} (Fig. 4B). The inferred binding order was slightly different for the competition experiment (SspB\textsuperscript{L1.48} \( > \) SspB\textsuperscript{L1.25} \( > \) SspB\textsuperscript{L1.91} \( > \) SspB\textsuperscript{L1.5}) than the degradation (SspB\textsuperscript{L1.48} \( > \) SspB\textsuperscript{L1.25} \( > \) SspB\textsuperscript{L1.5} \( > \) SspB\textsuperscript{L1.91}) experiment. These differences may arise because ssrA-tagged substrates, which contribute to binding (14), are bound to SspB in the degradation assays but are absent in the competition assays and/or because adaptor-mediated degradation requires both adaptor binding to ClpX and binding of the adaptor-bound ssrA substrate to the central pore of ClpX. Hence, some of the adaptor variants could bind well to ClpX on their own, but strain could be introduced when the ssrA tag of a bound substrate interacts with the ClpX pore (see “Discussion”). Nevertheless, it is clear that linker length affects the affinity of the SspB adaptor for ClpX. Moreover, the dimeric SspB\textsuperscript{L1.48}, SspB\textsuperscript{L1.25},

![FIGURE 4. Effects of SspB linker length on substrate delivery and ClpX binding.](image)

**TABLE 2**

Catalytic parameters of ClpXP degradation of GFP-DAS+4 in the presence or absence of SspB variants with different linker lengths

V\textsubscript{max}, K\textsubscript{m}, and Hill coefficient values are the averages of two determinations (error is estimated as \( \sqrt{\text{mean}^2 - \text{value}^2} \)).

| Adaptor | \( V_{\text{max}} \) (mM min\textsuperscript{-1} enz\textsuperscript{-1}) | Apparent K\textsubscript{m} (mM) | Hill coefficient | Degradation rate of 0.3 \mu M substrate (mM min\textsuperscript{-1} enz\textsuperscript{-1}) | -Fold increase with adaptor |
|---------|-----------------|-----------------|-----------------|-------------------------------|-----------------|
| None    | 0.18 \pm 0.11   | 34 \pm 30       | 1.1 \pm 0.2     | 0.0011                        | 62              |
| SspB\textsuperscript{L1.5} | 0.43 \pm 0.03 | 0.66 \pm 0.01   | 2.1 \pm 0.02 | 0.068                         | 109             |
| SspB\textsuperscript{L1.25} | 0.57 \pm 0.09 | 0.65 \pm 0.10   | 1.7 \pm 0.3     | 0.12                          | 191             |
| SspB\textsuperscript{L1.48} | 0.51 \pm 0.06 | 0.35 \pm 0.01   | 2.4 \pm 0.8     | 0.21                          | 38              |
| SspB\textsuperscript{L1.91} | 0.53 \pm 0.09 | 1.7 \pm 0.5     | 1.4 \pm 0.2     | 0.042                         | 2               |
Substrate Delivery by Variant SspB Adaptors

SspB14L, and SspB111L5 variants all bound ClpX more strongly than did the SspB monomer (Fig. 4B).

DISCUSSION

The results presented here show that a monomeric, single-tailed SspB variant stimulates ClpXP degradation of substrates bearing extended versions of the ssrA and DAS degradation tags. This finding demonstrates that there is no intrinsic requirement for bivalent tethering or a functional requirement for a second SspB subunit in adaptor-mediated substrate delivery to the ClpXP protease. As a consequence, we anticipate that other adaptor proteins may function by monovalent tethering of substrates to ATP-dependent proteases.

In agreement with previous studies (11), however, we observed little if any stimulation of ClpXP degradation of substrates with wild-type ssrA tags or non-extended DAS tags by single-tailed SspB variants. This result can be explained if close spacing of the SspB and ClpX binding sites in a degradation tag causes an unfavorable interaction that interferes with substrate delivery during monovalent and bivalent delivery. By this model, the interference cancels the positive effects of substrate tethering to ClpXP during monovalent delivery, and net stimulation of proteolysis occurs. During bivalent delivery, the unfavorable interaction is overcome by the second tethering contact between the adaptor and ClpX. As shown in Fig. 5 and discussed below, we believe that the second tether restricts the volume accessible to the substrate in ternary complexes and, therefore, facilitates engagement of the degradation tag by ClpXP. Experiments support the idea that SspB binding to wild-type ssrA tags interferes with ClpX recognition of the tag (10, 18). For example, tail-less SspB variants increase \( K_m \) for ClpXP degradation of GFP-ssrA from roughly 1 to 50 \( \mu M \). This inhibition is largely relieved for extended-tag GFP substrates (\( K_m \approx 2–3 \mu M \)). Moreover, in the presence of wild-type SspB, ClpXP degrades GFP substrates with extended LAA tags faster than GFP-ssrA, with improved degradation resulting from a reduction in \( K_m \). These results suggest that a modest clash (1–2 kcal/mol) occurs between SspB and ClpX when both proteins bind to the wild-type ssrA tag, but increasing the spacing between the SspB and ClpX binding sites in the tag relieves most of the unfavorable interactions (18). Our finding that monomeric SspB enhances ClpXP degradation of GFP substrates with extended but not normal-length tags is consistent with this model. Because we observe relief of inhibition by extension of the ssrA tag (which binds ClpX strongly) or extension of the DAS tag (which binds weakly), the clash must be present in both cases and, thus, be relatively independent of the strength of the ClpX-tag interaction. Moreover, because tag extension stimulates degradation mediated by singly tethered dimeric and monomeric SspB, the unfavorable interaction during delivery of substrates with normal-length tags probably occurs between ClpX and the SspB subunit that delivers the substrate to the ClpX pore.

For extended-tag substrates, monovalent versus bivalent tethering by SspB results in additional functional consequences. For example, ClpXP degradation of subsaturating GFP-DAS+4 is fastest with the wild-type SspB dimer, slower with the two-tailed monomer, and slower still with monomeric SspB (Table 1). These rate differences arise from changes in \( V_{\text{max}} \) and \( K_m \) for degradation. For example, \( V_{\text{max}} \) in the presence of the SspB dimer is roughly twice the value observed for the monomer, and \( K_m \) is about 15-fold lower for the dimer than the monomer. The doubled value of \( V_{\text{max}} \) does not reflect delivery by two subunits in the dimer as opposed to delivery by one subunit in the monomer. An SspB heterodimer with only one subunit able to bind substrate was shown to be as active as the wild-type SspB homodimer in delivering ssrA-tagged substrates to ClpXP (11). When we used a similar SspB heterodimer to deliver GFP-DAS+4, \( V_{\text{max}} \) for ClpXP degradation was 0.73 min\(^{-1}\) enzyme\(^{-1}\) (data not shown), slightly more than twice the value obtained using the SspB monomer.

Interestingly, higher \( V_{\text{max}} \) values for ClpXP degradation of GFP-DAS+4 are correlated with lower \( K_m \) values for all of the SspB variants shown in Fig. 3D. This behavior can be explained by the model shown in Fig. 5, which depicts engaged and unengaged adaptor-substrate-ClpX complexes. All possible binary interactions are made in engaged complexes (adaptor binds ClpX, substrate binds adaptor, ClpX binds substrate). The unengaged complex is stabilized by adaptor-substrate and ClpX-adaptor interactions only. The equilibrium between these states ([unengaged]/[engaged] = \( K_{EN} \)) will depend on the way in which the adaptor is tethered to the enzyme. For example, a substrate in a singly tethered complex could access a roughly spherical volume defined by the length of the tether, whereas a substrate in a doubly tethered complex would be constrained to a smaller ellipsoidal volume (Fig. 5). As a consequence, the entropic penalty associated with engagement would be larger for singly tethered than doubly tethered complexes. Because direct binding of the substrate to ClpX is a prerequisite for proteolysis, the maximum velocity of degrada-
Substrate Delivery by Variant SspB Adaptors

V}_{\text{max}} = V_{\text{limit}}/(1 + K_{\text{EN}}). Any change in tethering that decreased $K_{\text{EN}}$ would increase $V_{\text{max}}$ and also make binding stronger (reduce $K_m$) because the ternary complex would be stabilized by the additional contacts between ClpX and the degradation tag of the substrate. Thus, under conditions of substrate/adaptor saturation, we propose that engaged ternary complexes represent a higher proportion of total ternary complexes for dimeric SspB than monomeric SspB.

The equilibrium ratio of unengaged to engaged complexes will be a function of the tethering interactions and of the strength of the interaction between the substrate degradation tag and ClpX. The latter point is illustrated by the GFP-LAA + 4 substrate, which shows very weak adaptor-dependent variation in $V_{\text{max}}$ for ClpXP degradation (Fig. 3C; Table 1). In these cases, the LAA + 4 tag appears to bind ClpX strongly enough to ensure that the equilibrium strongly favors engaged ternary complexes both for dimeric and monomeric SspB variants. In the model of Fig. 5, favorable enthalpic contacts mediated by LAA + 4 tag binding to the ClpX pore would overcome the unfavorable entropy associated with engagement.

$V_{\text{max}}$ for ClpXP degradation of substrates with normal or extended ssrA tags is increased modestly by the SspB dimer, typically by 10–25% when compared with $V_{\text{max}}$ for the adaptor-independent reaction (Fig. 3C; Table 1; Refs. 9, 10, and 18). By contrast, the SspB dimer increases $V_{\text{max}}$ for ClpXP degradation by 3-fold or more for DAS + 4 substrates (Fig. 3D; Table 1; Ref. 17). Stably folded ssrA-tagged substrates are, on average, bound and released many times by ClpX before mechanical denaturation and degradation proceeds (30). This result suggests that the substrate has a low probability of denaturation during any single binding cycle and dissociates if denaturation does not occur. Because the maximal rates of adaptor-independent ClpXP degradation for DAS + 4 substrates are less than 20% of those of ssrA-tagged substrates (Table 1; Ref. 17), the probability of denaturation of a DAS + 4 substrate during any cycle appears to be significantly smaller than that of an ssrA-tagged substrate, presumably because weaker binding of the DAS + 4 tag to ClpX increases the probability of dissociation. When delivered by the SspB dimer, however, $V_{\text{max}}$ for ClpXP degradation of DAS + 4 substrates increases to roughly 50% of that of ssrA-tagged substrates (Table 1; Ref. 17). Thus, when the adaptor-tethered DAS + 4 tag disengages from the ClpX pore in the ternary complex, the rate at which it rebinds in the tethered reaction must be faster than would be possible in the bimolecular reaction without SspB tethering.

Varying the length of the linker region from 5 to 91 residues in the tails of the SspB dimer had modest effects on adaptor-mediated degradation of DAS + 4 substrates by ClpXP. $V_{\text{max}}$ for these reactions was within error for the three constructs with the longer linkers and was only reduced by ~20% for the variant with the 5-residue linker (Table 2). Substantial activity for another SspB variant with a very short linker was reported previously at one substrate concentration (10). Because varying the linker from 5 to 91 residues did not cause major changes in $V_{\text{max}}$, most ternary complexes formed by these dimeric SspB variants appear to be engaged.

$K_m$ for ClpXP degradation of GFP-DAS + 4 in the presence of the SspB linker-length mutants varied from a low of 0.35 μM for SspB$L^1L^4$ to a high of 1.7 μM for SspB$L^1L^9$, with intermediate values for SspB$L^1L^5$ and SspB$L^1L^6$. Because SspB$L^1L^5$ binds ClpX more tightly than the SspB monomer in competition experiments (Fig. 4B), it seems likely that the dimeric SspB$L^1L^5$ protein can make two tethering interactions with ClpX. However, the two XB motifs in SspB$L^1L^5$ would only be about 50 Å apart based on the crystal structure of the SspB dimer (12, 13), and thus, their tethering sites in the N-terminal domain of the ClpX hexamer would need to be roughly this distance apart. The N-domains are not observed in cryo-electron microscopy structures of ClpXP (31) and are probably somewhat flexible. Each N-domain is fused to the rest of ClpX near the periphery of the hexameric ring formed by the AAA + domains, at a position ~42 Å from the central pore (32). Moreover, the N-domain forms a homodimer (33). If these N-domain dimers were constrained to the outer edge of the ClpX ring, then it would be impossible for the XB segments of SspB$L^1L^5$ to contact two different N-domain dimers of ClpX, as has been proposed for wild-type SspB (11). If the N-domains were flexibly tethered to the ClpX ring, however, then they could move to positions that would allow double tethering of SspB$L^1L^5$. Indeed, the N-domain can cross-link to a site on the upper surface of the ClpX ring that is ~25 Å from the central pore (34), suggesting significant flexibility in its tethering to the rest of ClpX.

The results reported here have several implications for understanding SspB and other naturally occurring adaptor molecules and for the design of synthetic adaptors that mediate controllable degradation of specific cellular proteins. First, monovalent tethering of an adaptor-substrate complex to ClpXP can lead to substantial enhancement of substrate degradation. It remains to be determined whether an optimized monovalent adaptor could function as efficiently as a bivalent adaptor, but there is clearly no requirement for two tethering interactions between the adaptor and enzyme. Second, SspB tolerates major variations in the length of the linker that connects the XB motif and substrate binding domain with little loss of adaptor activity in the assays used here. This property, if generally applicable, should make it easier to design and evolve adaptors with some substrate-delivery function and then to optimize this activity in subsequent steps. Although long flexible tails like the one present in E. coli SspB are clearly not essential for adaptor-mediated delivery of substrates with ssrA-like degradation tags, longer tails might be important in allowing SspB to deliver other types of substrates to ClpXP. Third, adaptor-mediated increases in the rate of substrate degradation can arise both from a reduction in $K_m$ and from an increase in $V_{\text{max}}$. In our studies, adaptor-mediated increases in $V_{\text{max}}$ were only substantial for substrates with degradation tags that interacted poorly with ClpX. However, it is precisely substrates of this type for which degradation is likely to be adaptor-dependent (17).

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