Specificity in substrate and cofactor recognition by the N-terminal domain of the chaperone ClpX

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Notes:
Protein degradation is an essential component of biological regulation and protein quality control in all organisms. Cylindrical proteases, such as the proteasome, form large oligomers in which the proteolytically active sites are sequestered within an internal chamber. Access to the chamber is provided through narrow axial pores that exclude entry of large polypeptides and allow entry only of small peptides of ~30 residues in length (1). These cylindrical proteases typically form complexes with ATPases associated with various cellular activities (AAA\(^+\)) chaperones that denature substrates and then translocate them into the proteolytic chamber of the protease for degradation.

Clp\(X\) of *Escherichia coli* forms such a complex (2). Clp\(X\) is the AAA\(^+\) ATPase, and it belongs to the Clp/Hsp100 family, whereas Clp\(P\) is a cylindrical serine protease consisting of two rings with 7-fold symmetry (3). Clp\(X\) has an N-terminal domain whereas Clp\(P\) is a cylindrical serine protease consisting of two domains, a zinc-binding domain (ZBD) that forms dimers and a AAA\(^+\) ATP-binding domain that arranges into a hexamer. Analysis of the binding preferences of these two domains in Clp\(X\) revealed that both domains preferentially bind to hydrophobic residues but have different sequence preferences, with the AAA\(^+\) domain preferentially recognizing a wider range of specific sequences than ZBD. As part of this analysis, the binding site of the Clp\(X\) dimeric cofactor, Ssp\(B\), in ZBD in Clp\(X\) was determined by NMR and mutational analysis. The Ssp\(B\) C terminus was found to interact with a hydrophobic patch on the surface of ZBD. The affinity of Ssp\(B\) toward ZBD and the geometry of the Ssp\(B\)-ZBD complex were investigated by using the newly developed quantitative optical biosensor method of dual polarization interferometry. The data suggest a model for the interaction between Ssp\(B\) and the Clp\(X\) hexamer.

**Materials and Methods**

Clp\(XP\) of *Escherichia coli* forms such a complex (2). Clp\(X\) is the AAA\(^+\) ATPase, and it belongs to the Clp/Hsp100 family, whereas Clp\(P\) is a cylindrical serine protease consisting of two rings with 7-fold symmetry (3). Clp\(X\) has an N-terminal domain that forms a AAA\(^+\) domain (Fig. 6A, which is published as supporting information on the PNAS web site). The importance of the N-terminal domain of Clp\(X\) is evident from its absolute conservation across all sequenced genomes (4). Our group demonstrated that the N-terminal domain of Clp\(X\) is a C4-type zinc-binding domain (ZBD) that forms a very stable constitutive symmetric dimer in isolation and in full-length Clp\(X\). ZBD binds one Zn\(^{2+}\) per monomer (4, 5). Hydrophobic residues that form the interface between two ZBD monomers are highly conserved throughout the sequenced genomes (5). The AAA\(^+\) domain forms a hexameric ring complex in a nucleotide-dependent manner from which the ZBD protrudes (Fig. 6B). Clp\(X\) unfolds proteins and then feeds them into Clp\(P\) for degradation.

Two proteins that were initially recognized as Clp\(X\) substrates are the phage proteins \(\lambda\)O and MuA. More recently, ~50 endogenous *E. coli* Clp\(X\) substrates were identified through a proteomics approach (6). Clp\(XP\) has also been implicated in the degradation of C-terminally SsrA-tagged proteins. GFP with an SsrA sequence added to its C terminus has typically been used as a model substrate to study such tagged proteins (7). The Ssp\(B\) cofactor enhances the degradation efficiency of C-terminally SsrA-tagged proteins by Clp\(XP\) (8). The Ssp\(B\) polypeptide can be divided into a substrate-binding domain that forms a dimer and a C-terminal unstructured domain that binds to the ZBD in Clp\(X\) (9). It has been proposed that Ssp\(B\) binds to SsrA-tagged proteins forming a complex that subsequently binds to the Clp\(X\) hexamer (10). Hence, the binding of Ssp\(B\), loaded with substrate, to ZBD in Clp\(X\) functions to hold the substrate in place as the AAA\(^+\) domain of Clp\(X\) pulls the substrate, starting from the recognition motif, through Clp\(X\) and into Clp\(P\) for degradation. It is established that both C-terminal tails in the Ssp\(B\) dimer are required to enhance the degradation of SsrA-tagged substrates by Clp\(XP\) (11).

**Results**

ZBD and AAA\(^+\) Domains of Clp\(X\) Preferentially Bind to Hydrophobic Residues. To determine the roles of the ZBD and AAA\(^+\) (Clp\(X\)-ZBD) domains of Clp\(X\) in substrate and cofactor recognition, peptide array analysis was carried out to identify residues that are preferentially bound by these two domains of the chaperone (Fig. 7, which is published as supporting information on the PNAS web site). Purified ZBD or AAA\(^+\) domains were incubated with peptide arrays containing a total of 3,717 C-terminally attached peptides whose sequences were derived from 26 different proteins, some of which are known Clp\(X\) substrates (see Materials and Methods). The ZBD is dimeric under all conditions used (4, 5). Because substrates entering into Clp\(X\) hexamer will bind to the interior chamber of the AAA\(^-\) ring, and because the oligomerization of the AAA\(^-\) ring requires the presence of nucleotides (12), peptide array
analysis of the AAA\(^+\) domain was carried out in the absence of nucleotides to expose the putative polypeptide-binding sites that will be involved in substrate translocation and possibly unfolding. The binding of AAA\(^+\) to the peptide arrays in the presence of nucleotides was also carried out but was found to be significantly reduced (Fig. 8, which is published as supporting information on the PNAS web site); consequently, the proportion of binders was statistically insignificant, reflecting the fact that the hexamerized AAA\(^+\) ring can recognize only specific sequence tags such as the SsrA tag. Hence no further analysis of the data for AAA\(^+\) in the presence of nucleotides could be carried out.

Fig. 7 shows an example of the binding preferences of ZBD\(_2\) and AAA\(^+\) (in the absence of nucleotides) to arrays generated by using peptides derived from λO and MuA as detected by Western blot analysis. In general, ZBD\(_2\) and AAA\(^+\) bound different peptide sequences. For example, ZBD\(_2\) bound strongly to peptides derived from residues Glu\(^{49}\)-Met\(^{67}\) of λO, whereas AAA\(^+\) preferentially bound to peptides derived from residues Ile\(^{2}\)-Ser\(^{41}\), Pro\(^{215}\)-Leu\(^{241}\), and Lys\(^{283}\)-Leu\(^{299}\) of λO (Fig. 7). Because ZBD\(_2\) binds λO and is required for λO degradation by ClpXP (4), these results suggest that the region Glu\(^{49}\)-Met\(^{67}\) of λO is essential for the recognition of λO by ClpX (see below). ZBD\(_2\) and AAA\(^+\) also recognize different MuA peptide sequences. MuA is 663 aa long. Interestingly, the C terminus of MuA, which was proposed to be the ClpX-recognition motif (13), was only weakly bound by ZBD\(_2\) and only residues Glu\(^{665}\)-Lys\(^{669}\) were bound by the AAA\(^+\) domain (Fig. 7).

In analyzing the peptide array data, peptides were classified as binders of ZBD\(_2\) or AAA\(^+\) if their normalized percent intensity was ≥75% (see Materials and Methods); otherwise, they were classified as nonbinders. Separation of the peptides on the arrays into binder and nonbinder groups allowed for comparison of these groups with respect to amino acid occurrence. As can be seen in Fig. 1, both ZBD\(_2\) and AAA\(^+\) preferentially bound to sequences enriched in hydrophobic residues as well as in the positively charged lysine. Negatively charged residues were typically disfavored.

**ZBD\(_2\) and AAA\(^+\) Domains of ClpX Preferentially Bind to Distinct Sequence Patterns.** To determine whether the ClpX domains have distinct sequence-binding preferences, and because a large data set has been obtained, binder and nonbinder peptide sequences were submitted to Teiresias (14) to search for short consensus sequences that may be responsible for ZBD\(_2\) and AAA\(^+\) binding. The Teiresias output included sequence patterns that varied in length but were generally 3–6 aa long. Patterns were analyzed as described in Materials and Methods to rank the consensus sequences that occurred more frequently in binders than in nonbinders (Table 1).

The utility of this approach is demonstrated by the identification of several consensus sequences previously implicated as possible ClpX-recognition sequences. For example, the strongest binding of ZBD\(_2\) to the λO array occurs in the region Q\(^{48}\)FKVLLAILRKTYGWNKPM\(^{67}\) (Fig. 7A), and the patterns responsible for the binding may be [AG][ILMV][AG][KR][AG][K][ILMV][x][ILMV][x][ILMV] (ranked 7 and 14 in Table 1), corresponding to the λO region of A\(^{55}\)IL\(^{57}\) and K\(^{31}\)VLLAI\(^{58}\), respectively. As another example, it is known that SspB specifically binds ZBD in ClpX (4, 11). The binding is mediated by the last 10 residues in SspB (G\(^{156}\)GRPALRVK\(^{165}\)) (9, 15), which contain the ZBD\(_2\) consensus sequence ALxx[ILMV] (ranked 5 in Table 1). Other such consensus sequences from this analysis can be found for the putative substrates identified by Flynn et al. (6).

Patterns common to both ZBD\(_2\) and AAA\(^+\) binders are shown in bold in Table 1. In general, it is not surprising that there are such common patterns indicating an equal role of both domains in binding these patterns. It should be noted that more patterns were found for AAA\(^+\) binders than for ZBD\(_2\) binders (Table 1). This might indicate that ZBD\(_2\) exercises more specificity in substrate recognition, whereas the AAA\(^+\) domain more pro-

![Fig. 1. Analysis of ZBD- and AAA\(^+\)-binding preferences using peptide arrays. Shown are the ZBD\(_2\) and AAA\(^+\)-residue-binding preferences. The normalized percent occurrence for each amino acid was determined for both the binder (black bars) and nonbinder (gray bars) groups. Asterisk indicates that the percent occurrence for each amino acid was determined for both the binder and nonbinder peptide sequences.](Image)
spB154–165. Fig. 2. Binding of spB154–165 to ZBD2 as monitored by NMR. (A) A heteronuclear sequential quantum correlation spectrum of 0.25 mM 15N-labeled ZBD2 in the absence (black) or presence of 5 mM of spB154–165 (red). * chemical shifts that disappear upon addition of peptide. Insets show the NH chemical shift changes of four residues in ZBD2 in the presence of 0 (black), 0.5 mM (blue), 2.5 mM (green), and 5 mM (red) of spB154–165. (B) Shown are the chemical shift changes \[ \Delta \delta^N = \frac{1}{2} \Delta \delta_{\text{H}} + \Delta \delta_{\text{15N}} \] in the presence of 5 mM spB154–165. *, residues whose chemical shifts disappeared upon addition of 5 mM spB154–165. (C) Ribbon and surface representation of ZBD dimer (Protein Data Bank ID code 1OVX). (5). Middle and Bottom are rotated 90° along the horizontal axis with respect to Top and Middle, respectively. In column 1, helices are colored blue, strands are red, and Zn(II) atoms are shown as pink spheres. In column 2, the electrostatic potential surface of ZBD2 is shown with negatively charged, positively charged, and hydrophobic surfaces in red, blue, and gray, respectively. In column 3, residues for which \( \Delta \delta^N > 400 \) Hz and whose chemical shifts disappeared in the spB154–165 titration experiments are colored purple and green, respectively. All structures were drawn by using PyMOL (http://pymol.sourceforge.net).

To verify some of the results of the peptide array analysis, the following experiments were carried out. Initially, the ClpXP-mediated degradation of \( \alpha \) was carried in the presence of 50 times excess of the peptides \( \alpha \delta^N_{49–63} \), \( \alpha \text{MuA}^N_{653–663} \), or spB154–165 (Fig. 9A, which is published as supporting information on the PNAS web site). According to the peptide array analysis, ZBD2 bound to peptides corresponding to \( \alpha \delta^N_{49–63} \) but not to those corresponding to \( \alpha \text{MuA}^N_{653–663} \) (Fig. 7A). Consistent with these results, excess of \( \alpha \delta^N_{49–63} \) peptide slowed down the ClpXP-mediated degradation of \( \alpha \), whereas \( \alpha \text{MuA}^N_{653–663} \) did not affect the rate of degradation. In addition, the C terminus of spB, spB154–165 peptide, significantly slowed down \( \alpha \delta^N_{49–63} \) degradation (Fig. 9A), suggesting that \( \alpha \delta^N_{49–63} \) and spB have similar or adjacent binding sites on ZBD in ClpX.

In another set of experiments, the peptides IYYT-GESLKA (IYY) and DVRGLVVISARKGE (DVG) were added at the N terminus of a sequence consisting of 6xHis tag followed by a tobacco etch virus recognition sequence and then GFP to form the constructs IYY-GFP and DVG-GFP, respectively. In the peptide array experiments, IYY and DVG peptides were preferentially bound by ZBD2 and AAA\(^{C} \), respectively. Consistent with those experiments, ELISA analysis confirmed that ZBD2 preferentially bound IYY-GFP, whereas AAA\(^{C} \) preferentially bound DVG-GFP (Fig. 9B). However, it should be noted that neither IYY-GFP nor DVG-GFP were unfolded or degraded by ClpXP (data not shown), indicating that, whereas some sequences are required for recognition by ClpX, other additional sequences might be required for unfolding and degradation.

**SpB C Terminus Binds a Hydrophobic Patch on the Surface of ZBD2.** The peptide array analysis revealed that ZBD in ClpX might recognize a limited set of specific sequence patterns. To understand how ZBD2 recognizes such sequence patterns, we endeavored to map the binding site for spB\(^{C} \) cofactor on ZBD2. To this end, a series of \( ^1\text{H}, ^{15}\text{N} \) heteronuclear sequential quantum correlation spectra were recorded of a uniformly \( ^{15}\text{N} \)-labeled ZBD2 in the presence of increasing concentrations of spB154–165 (Fig. 2A) or spB\(^{C} \) (data not shown). spB154–165 consists mostly of hydrophobic residues (P154RGGRPALRVVK165). The NH chemical shift assignments that we determined (5) were used. Upon addition of spB154–165, the chemical shifts of several residues systematically moved as the peptide concentration was increased (Fig. 2A), and saturation was typically reached at peptide to ZBD2 concentration ratio between 2.5 and 5 (Fig. 2A Inset).

The NH groups of residues Cys17, Gln21, Gly30, Tyr34, Ile35, and Ile46 in ZBD2 (0.25 mM) shifted by \( >400 \) Hz (\( \Delta \delta^N \); see Materials and Methods) in the presence of 2.5 mM of spB154–165 (Fig. 2B). Because the maximum shift observed was \( \approx 800 \) Hz (Fig. 2B), chemical shifts more than half the maximum, i.e., \( >400 \) Hz, were arbitrarily considered to be significant. The chemical shifts of three ZBD residues (Lys26, Leu27, and Ala29) disappeared upon addition of spB peptide (marked by an asterisk in Fig. 2A and B). The same phenomenon was observed upon addition of spB\(^{C} \).
of the ZBD2 box (Fig. 2 (top view), two identical hydrophobic surfaces exist on both sides that the side hydrophobic surfaces of ZBD2 are the primary due that Cys17, Gln21, Leu27, Ala29, Gly30, Tyr34, and Ile46 are part of surface is present on the bottom (Fig. 2 tails of SspB2.

Measuring the Binding Affinity and Geometry of the Interaction of SspB2 to ZBD2. To further support the conclusions made above, isothermal titration calorimetry (ITC) experiments were carried out at 20°C to determine the binding constant of SspB154–165 peptide to the different ZBD2 mutants. ZBD2 WT bound SspB154–165 with a K_d of 34.13 ± 5.94 μM and n = 0.97 ± 0.07 (Fig. 12A and B, which is published as supporting information on the PNAS web site), suggesting that only one site on the hydrophobic surfaces of ZBD2 can be saturated by the peptide. This could indicate that the binding of SspB154–165 to one surface prevents interactions with the second surface as a result of some slight but critical conformational changes. Other interpretations are also possible. The binding of SspB154–165 to ZBD2(Y34W) gave a K_d of 30.49 ± 4.09 μM and n = 3.08 ± 0.04 (Fig. 12C and D). Suggesting that the mutation of Y34 to Trp allowed for the binding of the peptide to more than one site on the hydrophobic surfaces of ZBD(Y34W). Finally, as expected, ZBD2(F16W) and ZBD2(A29N) did not show any significant binding to SspB154–165 (K_d > 200 μM; data not shown). Hence, these results, combined with the NMR and mutational analyses, strongly suggest that residues F16 and A29 are required to preserve the hydrophobic surfaces of ZBD2, and that the binding mainly occurs through the C termini of the SspB dimer.

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independent binding events gave a very good fit to the experimental points (Fig. 4B). The strong association reaction had a $K_d$ of 0.85 μM (SpB2 to ZBD2) with a final thickness of 3.3 nm at saturation (Figs. 4B and 13C). The thickness obtained agrees with the expected dimension of a layer of SpB dimers, placed on their sides, on top of the ZBD dimers (Fig. 13C Upper) (5, 16, 18). The weak association reaction had a $K_d$ of 11.0 μM (SpB2 to ZBD2) and a final thickness of 7.6 nm (Figs. 4B and 13C). The data suggest that the SpB dimer in this case is oriented vertically upon binding to ZBD2 (Fig. 13C). Also, at saturation, the stoichiometry of the interaction between SpB2 and ZBD2 was found to be 1:1. It was previously reported that the binding affinity of SpB154–165 to ClpX hexamer, SpB154–165 to ZBD dimer, and SpB dimer to ClpX hexamer has a $K_d$ of 22.8, 20.0, and 1.3 μM, respectively (10, 11, 15), which are close to the values obtained in our analysis (Fig. 4C).

Using this technique, it is possible to estimate the area occupied by molecules on the surface of the chip. For the high-affinity interaction, SpB2 occupies an area corresponding to twice that occupied by a single ZBD2 (Fig. 13D). On the other hand, for the low affinity interaction, SpB2 occupies an area corresponding to a single ZBD2 (Fig. 13D). We propose that the strong binding interaction is due to the association of the SpB dimer to two ZBD dimers by using both C termini of SpB2, whereas the weak association interaction is due to the association of SpB2 to one ZBD2 through only one C terminus.

Discussion

Rigorous analysis of the binding of ZBD and AAA$^+$ domains of ClpX to a large peptide library allowed us to identify novel binding preferences for these two ClpX domains. Although ZBD and AAA$^+$ domains favor binding to hydrophobic residues, these domains preferentially recognize different amino acid sequences. This suggests that the ZBD and AAA$^+$ domains bind to different classes of polypeptides and, hence, “filter” the interaction between ClpX and its putative substrates.

The binding of SpB2 to ZBD2 occurs mainly through the interaction of the C terminus of SpB2 to hydrophobic patches present on the surface of ZBD2, as determined by NMR and mutagenesis analyses (Figs. 2 and 3). The interaction is significantly enhanced by the binding of two C-terminal tails of SpB2 to ZBD2 (Fig. 4C). Interestingly, residue Leu$^{161}$ at the C terminus of SpB is highly conserved and is essential for the
enhancement of the degradation of SsrA-tagged proteins by ClpXP (15). Therefore, it would be reasonable to propose that Leu<sup>140</sup> of SspB<sub>1</sub> is one of the C-terminal residues that directly interact with the hydrophobic patch on ZBD<sub>2</sub> surface.

In principle, three SspB dimers can bind to one ClpX hexamer. However, it has been experimentally determined that only one SspB<sub>2</sub> binds to a ClpX hexamer at a given time (19). We had proposed earlier that the ZBD domains in ClpX hexamer might come together to form a trimer of dimers at one stage during the chaperone functional cycle (5). Fig. 5 shows the four possible modes by which two tails of SspB<sub>2</sub> might interact with the ZBD trimer of dimers. In Fig. 5, it is assumed that the ClpX AAA<sup>+</sup> ring is below the ZBD<sub>5</sub>s. Based on our binding analysis (Figs. 4, 12, and 13) and on published literature (10, 11, 15), the binding mode of Fig. 5D in which the two tails of SspB<sub>2</sub> interact with the same ZBD<sub>2</sub> is unlikely to occur, especially that an SspB tail can reposition the other two tails of SspB<sub>2</sub> in one of the other binding modes by which two tails of SspB<sub>2</sub> might interact with the ZBD <sup>12</sup>, and 13) and on published literature (10, 11, 15), the binding 

However, it has been experimentally determined that only one SspB<sub>2</sub> binds to only one site on ZBD<sub>2</sub> according to ITC measurements (Fig. 12). In Fig. 5A and C, the two tails of SspB<sub>2</sub> do not interact “symmetrically” with the ZBD surface and would have to be 

likely. This mode of binding would also be in agreement with our 

recently published data suggesting a nucleotide-dependent block 

movement of the ZBD<sub>2</sub> toward the AAA<sup>+</sup> ring in ClpX (20).

In the model of Fig. 5B, one tail binds to the top of one ZBD<sub>2</sub>, whereas the other tail binds to the bottom of the second ZBD<sub>2</sub>. The third ZBD<sub>2</sub> can be prevented from interacting with other cofactors or substrates by the folded domain of the bound SspB<sub>2</sub>. The possible movements of the ZBD<sub>2</sub> can then drive the bound SspB<sub>2</sub> closer to the AAA<sup>+</sup> ring to deliver the SsrA-tagged substrate, whereas the tails of SspB<sub>2</sub> reposition the other two ZBD<sub>2</sub>s away from the entry pore. This model implies that the enhancing activity of SspB<sub>2</sub> is due in part to its ability to direct the movement of ZBD<sub>2</sub> and to regulate the delivery of tagged substrates in addition to increasing the local concentration of those substrates near ClpX.

**Materials and Methods**

**Protein Purification and Peptide Synthesis.** Proteins were expressed, purified, and manipulated as described (4). Peptides were purchased from Dalton Chemical Laboratories (Toronto, ON, Canada). CD measurements of 15 μM ZBD<sub>2</sub> WT and mutants were carried out in buffer A (25 mM Tris-HCl, pH 8/150 mM NaCl/1 mM DTT) by using Jasco (Easton, MD) J-810. Degradation assays were typically carried out in buffer B (25 mM Hepes, pH 7.5/5 mM MgCl<sub>2</sub>/5 mM KCl/0.03% Tween-20/10% glycerol), as described (4).

**Peptide Array Experiments.** Peptide arrays were prepared by using an AutoSpot ASP 222 spot synthesizer (intavis AG) according to a standard spot synthesis protocol. Each peptide was 13 aa long, with a 17-frame shift of 2 aa along the protein sequence, for a total of 3,717 peptides tested. Three independent peptide array incubation experiments were analyzed by using a procedure similar to that of Rüdiger et al. (21). Further details are given in Supporting Text, which is published as supporting information on the PNAS web site.

**NMR Spectroscopy.** Uniformly <sup>15</sup>N-labeled ZBD<sub>2</sub> was prepared by growing the *E. coli* strain BL21 gold in minimal media containing <sup>15</sup>NH<sub>4</sub>Cl. The NMR sample concentration was typically 0.25 mM of ZBD<sub>2</sub> in buffer C (20 mM sodium phosphate, pH 7.6/150 mM NaCl/10% D<sub>2</sub>O). NMR spectra were recorded at 20°C on a 500 MHz Varian Spectrometer. <sup>1H</sup> and <sup>15</sup>N heteronuclear single quantum correlation experiments were carried out in the absence or presence of SspB<sub>2</sub> full length or SspB<sub>154–165</sub> peptide. Changes in the chemical shifts of ZBD<sub>2</sub> NH groups (Δδ<sub>NH</sub>) were derived from (∆δ<sub>B</sub> + (∆δ<sub>B</sub> + ∆δ<sub>CH</sub>))<sup>1/2</sup>, where Δδ is the chemical shift change expressed in Hz.

**Measuring Binding Affinities and Geometries.** ITC experiments were performed at 20°C. Twenty-nine 10-μl injections of 1.1 or 2 mM SspB<sub>154–165</sub> were added to 1.4 ml of 70 μM ZBD<sub>2</sub> or ZBD<sub>2</sub> mutants. Peptide and proteins were resuspended in buffer D (50 mM potassium phosphate, pH 8.7/75 mM NaCl/1 mM DTT). The thermograms were fit to a one-site model by using Origin 7 software (OriginLab, Northampton, MA). ITC experiments were performed by using a Microcal (Amherst, MA) VP-ITC and were repeated three times. Other binding experiments were performed by using an AnaLight Bio200 dual waveguide interferometer instrument from Fairfield (16). Experiments were performed in buffer D at a flow rate of 0.05 ml/min. ZBD<sub>2</sub> (0.5 mg/ml) was crosslinked to both channels of the sensor chip by incubating with [Bis(sulfosuccinimidy)] suberate (BS<sub>3</sub> (Pierce, Rockford, IL). Free BS<sub>3</sub> was blocked by using 10 mg/ml glycosamine. After the establishment of a stable buffer baseline, SspB<sub>154–165</sub> or SspB was injected into one channel, whereas the second channel was used as a reference.

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Supporting Materials and Methods

**Peptide Array Analysis.** Peptides were derived from λO (Swiss-Prot # P03688), MuA (Swiss-Prot # P07636), SspB (Swiss-Prot # P0AFZ3), RepA (Swiss-Prot # P06019), ClpX (Swiss-Prot # P0A6H1), casein (Swiss-Prot # P02662), MetK (Swiss-Prot # P0A817), Dps (Swiss-Prot # P0ABT2), MDH (Swiss-Prot # P61889), LexA (Swiss-Prot # P0A7C2), MiaA (Swiss-Prot # P16384), Hsp82 (Swiss-Prot # P02829), hemagglutinin precursor (Swiss-Prot # P03438), alpha-1-antitrypsin precursor (Swiss-Prot # P01009), H-2 class I histocompatibility antigen D-B alpha chain precursor (Swiss-Prot # P01899), CD74 antigen (Swiss-Prot # P04233), spike glycoprotein precursor (Swiss-Prot # P03522), Cd3e (Swiss-Prot # P22646), Porcine citrate synthase (Swiss-Prot # P00889), PI3-kinase p85-β subunit (Swiss-Prot # O08908), UmuD (Swiss-Prot # P0AG11), σ^S (Swiss-Prot # P13445), Phd (Swiss-Prot # Q06253), λW (Swiss-Prot # P68660), eRF2 (Swiss-Prot # P05453), and acid phosphatase precursor (Swiss-Prot # P08091) sequences.

10 µg/mL of purified ZBD₂ or AAA⁺ were incubated in buffer E (25 mM TrisHCl, pH 7.5, 150 mM NaCl, 0.1% Tween, 0.1% BSA, and 0.002% thimerosal) with peptide arrays previously blocked with 2% BSA in buffer E for 1 hour. The bound protein was transferred to a nitrocellulose membrane using a Hoefer TE Series semi-dry blotter (Amersham Biosciences), at 0.75 mA/cm² for 30 minutes using buffer F (25 mM TrisHCl, pH 8.3, 192 mM glycine, 0.1% SDS, and 20% methanol). ZBD₂ and AAA⁺ were detected using rabbit polyclonal α-ClpX antibodies, and then visualized on film using Protein LA-Peroxidase (Sigma) and ECL substrate (Amersham Biosciences). Three independent peptide array incubation experiments were analyzed.

After scanning the film, spot volumes or intensities were measured using ImageQuant 5.0 (Molecular Dynamics) software. All of the spot volumes were then averaged and normalized.
Peptides that had spot intensities greater than 75% were considered binders, otherwise they were classified as non-binders. The number of each type of amino acid was counted in the binder and non-binder sequences and the percent occurrence of each amino acid in each group (binders and non-binders) and the normalized percent occurrences were calculated, as follows:

\[
\text{% occurrence in binders} = \frac{\text{no. of aa } 'x' \text{ in binders}}{\text{total no. of aa in binders}} \times 100
\]  

(1)

Normalized % occurrence in binders = \( \frac{\% \text{ occurrence of aa } 'x' \text{ in binders}}{\% \text{ occurrence of aa } 'x' \text{ on array}} \times 100 \)  

(2)

The z-test for comparing two proportions was used to determine if the percent occurrences of the specific amino acids in the binder or non-binder groups differed from their respective occurrences on the array at the 95% significance level.

The software Teiresias was used to search for recurring sequence patterns in the binder and non-binder peptide groups. For each sequence pattern, the frequencies of the pattern in the binder (F_B) and non-binder (F_NB) groups were determined by dividing the number of peptides in each group that contain the pattern by the total number of peptides in that group. The ratio R (R = F_B/F_NB) was calculated. Patterns that pass the z-test at 99.9% confidence level and that fulfill the criteria F_B \geq 0.045 and R \geq 4.0 were selected. The most general form of a sequence pattern is shown (Table 1). For example, a pattern will be reported as having [ILMV] for an amino acid instead of the version that specifically shows I, L, M, or V if both the general and specific forms of the pattern satisfy one of the three criteria listed above. The Teiresias and statistical analyses were implemented in a program developed in-house termed Sequence Array Analyzer.

**ELISA assay.** The wells of a 96-well plate were coated with DEA-GFP, DVG-GFP, IYY-GFP, or GFP-SsrA (100 μL, 100 μg/mL) in 20 mM Na_2CO_3, pH 9 for 1 h at 37°C. The wells were then washed with buffer I (25 mM TrisHCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) and
incubated with 200 μL of 5% milk protein in buffer I for an additional hour at 37°C. The wells were then washed with buffer J (25 mM Hepes, pH 7.5, 150 mM KCl, 25 mM NaCl, 10 mM MgCl₂, 2.5% glycerol, 0.1 mM EDTA, and 0.1% Tween-20) plus 1 mM DTT and incubated with 25 μM of ZBD₂ or 1 μM AAA⁺ for 1 h (total volume 100 μL). This step and all subsequent steps were performed at room temperature. Wells were washed with buffer J (200 μL, 3 times), incubated with 100 μL of a 1:3000 dilution of anti-ClpX serum for 1 h, washed, and then HRP-conjugated Protein A (1:10,000 dilution, 100 μL) in buffer J was added to the wells and incubated for 1 h. The wells were washed and 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate (Sigma T0440) was added. The reaction was allowed to proceed for 30 minutes and the absorbance was measured at 650 nm using a SPECTRAmax 340PC plate reader (Molecular Devices).

**Legends for Supporting Figures**

**Supporting Fig. 6. Structural model of the ClpXP Complex.** (A) Shown are the domain boundaries of ZBD and AAA⁺ in ClpX. (B) A model of the ClpXP complex. The structures shown are based on the solved structure of *E. coli* ClpP (1, 2), a model for the hexamer of the AAA⁺ domain of *E. coli* ClpX based on the solved monomeric AAA⁺ domain of *H. pylori* ClpX (3) and the hexameric *E. coli* HslU structure (4), and on our proposed trimer-of-dimers model for the ZBD of *E. coli* ClpX (5). Note that ClpX oligomers can bind to both ends of the ClpP cylinder. Structures were drawn using PyMOL (http://pymol.sourceforge.net).

**Supporting Fig. 7. Binding of ZBD₂ and AAA⁺ to the arrays of λO and MuA.** (A) Purified
ZBD₂ or (B) AAA⁺ in the absence of nucleotides were incubated with peptide arrays containing a total of 3717 13-mer peptides spanning the sequences of 26 proteins, with a frame shift of two amino acids. The arrays for λO and MuA are shown as examples. Labels to the left give the residue number of the N-terminal amino acid of the first peptide of each row, while labels to the right give the number of the last peptide in each row.

Supporting Fig. 8. Effect of ATP on the binding of AAA⁺ to the peptide array. Purified AAA⁺ was incubated with peptide arrays in the absence (top panel) or presence (bottom panel) of 5 mM ATP. The array of H-2 class I histocompatibility antigen D-B alpha chain precursor [H-2D(B)] is shown as an example. Labels to the left give the residue number of the N-terminal amino acid of the first peptide of each row, while labels to the right give the number of the last peptide in each row.

Supporting Fig. 9. Peptide-ClpX interaction. (A) The ClpP-dependent degradation of λO (3.9 μM monomer) mediated by ClpX (1 μM monomer) and ClpP (1.2 μM monomer) was monitored by SDS-PAGE in the absence of peptide (top lane), in the presence of 50 μM λO⁴⁹–⁶³ (second lane), 50 μM MuA⁶⁵³–⁶⁶³ (third lane), or 50 μM SspB¹⁵⁴–¹⁶⁵ (bottom lane) peptides. Aliquots were removed from the degradation mixture at the indicated time points. The chaperone was the last component added to the reaction mixture. (B) ELISA assays for the binding of 25 μM ZBD₂ or 1 μM AAA⁺ (monomer concentration), respectively, to 100 μg/mL of N-terminally tagged GFP or GFP-SsrA. Data points are the average of two experiments and normalized to GFP-SsrA absorbance. DVG-GFP and IYY-GFP correspond to His-tagged GFP with the peptides DVGVLVISARKGE (DVG) and IY YITGESLKA VE (IYY), respectively, introduced at the N-
terminus. Based on Table 1, the DVG peptide contains patterns ranked 3 and 7 preferentially bound by ZBD$_2$ and patterns ranked 4, 6, 9, 12, 16, and 18 preferentially bound by AAA$^+$. The IYY peptide contains patterns ranked 1, 2, 4, 8, and 15 preferentially bound by ZBD$_2$ and the pattern ranked 1 preferentially bound by AAA$^+$.

**Supporting Fig. 10. Mutational analysis to determine the SspB$_2$ binding site on ZBD in ClpX.** The ClpP-dependent degradation of GFP-SsrA (3.9 µM monomer) mediated by ClpX or different ClpX mutants (each at 1 µM monomer) and ClpP (1.2 µM monomer) are shown in the presence of 0 (a), 0.025 µM (b), and 0.165 µM (c) of SspB$_2$.

**Supporting Fig. 11. Stability of different ZBD$_2$ mutants.** (A) CD spectra at 10°C of ZBD$_2$ WT and different mutants (30 µM). (B) Thermal denaturation curves monitored by CD at 220 nm. (C) The oligomeric states of the ZBD$_2$ mutants were analyzed by size exclusion chromatography on a Superdex 200 HR 10/30 column. Molecular mass standards, in kDa, are shown along the top. ‘VV’ refers to void volume. Due to the column resolution and the structure of wildtype ZBD$_2$, the protein elutes near the 29 kDa marker even though the molecular mass of the dimer is about 14 kDa.

**Supporting Fig. 12. Determination of the binding affinity of SspB$^{154-165}$ to ZBD$_2$ WT and ZBD$_2$(Y34W) using ITC.** Shown are raw ITC (A and C) and integrated heat data (B and D) of addition of SspB$^{154-165}$ to ZBD$_2$. Titrations were done at 20°C. Solid lines in (B) and (D) represent the fit to a model of a single type of n identical and independent binding sites. The n and K$_d$ resulting from the fits are given.
Supporting Fig. 13. Raw data and model of the binding affinity of SspB$_2$ and SspB$^{154-165}$ to ZBD$_2$ using DPI. Shown are raw sensor data of addition of free SspB$_{154-165}$ (A) or free SspB$_2$ (B) to immobilized ZBD$_2$. T, D, and M refer to thickness, density, and mass, respectively. Different additions of SspB$_{154-165}$ or SspB$_2$ are indicated with dashed lines. The concentrations of injected SspB$_{154-165}$ or SspB$_2$ are shown on the top x-axis. (C) Shown are examples of the binding mode of SspB$_2$ to ZBD$_2$ drawn to scale. ZBD$_2$ on the sensor chip is drawn in light grey with the charged surface facing the chip. SspB$_2$ is shown as 2 triangles with the C-terminal tails binding to ZBD$_2$. Numbers on the left represent the thickness resulting from SspB$_2$ binding at saturation. Thickness measurements have an estimated error of 5 – 10%. Other modes of binding cannot be excluded. (D) The areas occupied by immobilized ZBD$_2$ and by bound SspB$_2$ in the low and high affinity interactions are given.
References

Supporting Figure 6

A

B
Supporting Figure 7
Supporting Figure 9

A

No peptide

$\lambda O$

$\lambda O^{49-63}$

MuA$^{653-663}$

SspB$^{154-165}$

Time (min)

B

Normalized absorbance change at 650 nm

- DVG-GFP
- IVY-GFP
- GFP-SsrA

Normalized absorbance change at 650 nm

ZBD$_2$

AAA$^+$
Supporting Figure 10
Supporting Figure 11
Supporting Figure 12

A

$$\mu \text{cal/sec}$$

WT

B

$$\text{Kcal/mole}$$

n: 0.97 ± 0.07
$$K_d: 34.13 \pm 5.94 \text{ µM}$$

WT

C

0 1 2

D

0 1 2 3 4 5 6 7

n: 3.08 ± 0.04
$$K_d: 30.49 \pm 4.09 \text{ µM}$$

Molar Ratio [SspB$^{154-165}$]/[ZBD$_2$]
Supporting Figure 13

A. [SspB$^{154-165}$] injected (µM)

B. [SspB$_2$] injected (µM)

C. High affinity

Low affinity

D. 

<table>
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<th>ZBD$_2$</th>
<th>SspB$_2$</th>
<th>SspB$_2$</th>
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<td>Area ($\text{Å}^2$ molecule$^{-1}$)</td>
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