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Examination of the Polypeptide Substrate Specificity for *Escherichia coli* ClpA

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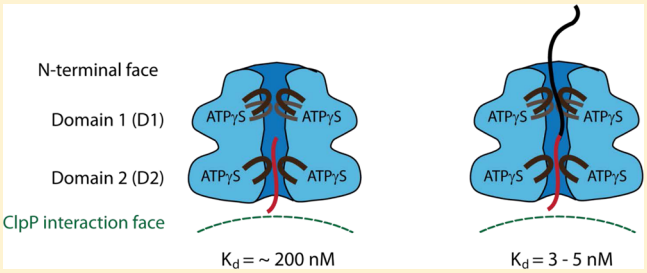
Supporting Information

ABSTRACT: Enzyme-catalyzed protein unfolding is essential for a large array of biological functions, including microtubule severing, membrane fusion, morphogenesis and trafficking of endosomes, protein disaggregation, and ATP-dependent proteolysis. These enzymes are all members of the ATPases associated with various cellular activity (AAA+) superfamily of proteins. *Escherichia coli* ClpA is a hexameric ring ATPase responsible for enzyme-catalyzed protein unfolding and translocation of a polypeptide chain into the central cavity of the tetradecameric *E. coli* ClpP serine protease for proteolytic degradation. Further, ClpA also uses its protein unfolding activity to catalyze protein remodeling reactions in the absence of ClpP. ClpA recognizes and binds a variety of protein tags displayed on proteins targeted for degradation. In addition, ClpA binds unstructured or poorly structured proteins containing no specific tag sequence. Despite this, a quantitative description of the relative binding affinities for these different substrates is not available. Here we show that ClpA binds to the 11-amino acid SsrA tag with an affinity of 200 ± 30 nM. However, when the SsrA sequence is incorporated at the carboxy terminus of a 30−50-amino acid substrate exhibiting little secondary structure, the affinity constant decreases to 3−5 nM. These results indicate that additional contacts beyond the SsrA sequence are required for maximal binding affinity. Moreover, ClpA binds to various lengths of the intrinsically unstructured protein, α-casein, with an affinity of ∼30 nM. Thus, ClpA does exhibit modest specificity for SsrA when incorporated into an unstructured protein. Moreover, incorporating these results with the known structural information suggests that SsrA makes direct contact with the domain 2 loop in the axial channel and additional substrate length is required for additional contacts within domain 1.



ATP-dependent proteases are essential components of protein quality control pathways in all organisms.[1,2] Such ATP-dependent proteases share a common architecture of a ring-shaped AAA+ protein unfoldase flanking one or both ends of a barrel-shaped protease with protease active sites sequestered from solution.[3,4] *Escherichia coli* ClpAP is a prokaryotic ATP-dependent protease composed of the hexameric protein unfoldase ClpA and the tetradecameric serine protease ClpP.

ClpA is a molecular chaperone that couples the energy from ATP binding and hydrolysis to enzyme-catalyzed protein unfolding and translocation.[5] When ClpA is associated with the serine protease, ClpP, ClpA enzymatically unfolds proteins and translocates them through its axial channel and into the central cavity of the tetradecameric ClpP for proteolytic degradation.[6] In the absence of ClpP, ClpA remodels an inactive dimer of RepA into active monomers.[7] Thus, ClpA also functions as a molecular chaperone in the absence of the proteolytic component.

ClpA binds and translocates a variety of substrates displaying short polypeptide sequences into the proteolytic cavity of ClpP. One such sequence is the 11-amino acid SsrA sequence, which is cotranslationally incorporated at the carboxy terminus of partially synthesized proteins on stalled ribosomes.[8] In addition to such tagged proteins, ClpAP has also been shown to degrade unfolded proteins containing no specific binding sequence.[9] However, a quantitative thermodynamic study of the relative affinities of various substrates binding to ClpA has not been presented. This is important because ClpA operates both in protein remodeling reactions by itself and in proteolytic degradation when associated with ClpP. Accordingly, how the enzyme discriminates between substrates in the crowded environment of the cell and remodels or proteolytically degrades remains an unanswered question.

Steady-state degradation of SsrA-tagged proteins catalyzed by ClpAP has been examined by incorporating the SsrA sequence into green fluorescent protein.[5,10,11] From these studies, a Michaelis constant (K_m) of 1.5−1.7 μM has been reported at temperatures of 23 and 30 °C. Similarly, a K_m of ∼1.2 μM at 37 °C for steady-state degradation of the intrinsically unstructured protein (IUP), α-casein, has also been reported.[12] Comparison of the K_m values for these different substrates might suggest that there is little substrate specificity for the SsrA tag compared to the unstructured protein. However, to quantitatively examine

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the binding specificity, a comparison of the equilibrium binding constant is required because it is possible that the Michaelis constant is not equal to the binding equilibrium constant. Moreover, the reported K_m values have been determined under different solution conditions and at various temperatures.

To date, the only binding equilibrium constant that has been reported is that for binding of ClpA to the 11-amino acid SsrA polypeptide in the absence of the proteolytic component, ClpP, which was reported to be 100−200 nM.[13] Consistent with the possibility that the Michaelis constant is not the same as the binding constant, the reported K_d for the 11-amino acid polypeptide is approximately 1 order of magnitude tighter than the K_m for an SsrA-tagged GFP.

An alternate explanation for this discrepancy between the K_d for ClpA binding the 11-amino acid SsrA polypeptide and the K_m for ClpAP-catalyzed degradation of an SsrA-tagged GFP is that ClpA may bind an SsrA substrate with an affinity different from that of ClpAP, i.e., ClpA associated with ClpP. In other words, the proteolytic component, ClpP, may allosterically influence the SsrA binding properties of ClpA. However, it is not possible to determine a K_m for ClpA in the absence of ClpP because ClpA does not covalently modify the substrate that it translocates. Thus, to answer the question of allostery, there is a need to compare the polypeptide binding equilibrium constant of ClpA to that of ClpAP.

The hypothesis that ClpP may impact the affinity of binding of the peptide to ClpA is not without precedence. We have shown that ClpA catalyzes polypeptide translocation using a different mechanism when bound to ClpP than when alone.[14,15] Thus, information about the mechanism of ClpA binding and translocation from the observation of proteolytic degradation catalyzed by ClpP may not represent the mechanism used by ClpA in the absence of the proteolytic component.

Here we report the results from quantitative anisotropy titrations performed to examine binding of ClpA to both SsrA-tagged substrates and unstructured proteins lacking any known tag sequence for ClpA binding. Similar to what has been reported by others,[13] we determined a binding dissociation equilibrium constant (K_d) of ∼200 nM for the 11-amino acid SsrA tag. Strikingly, when the 11-amino acid substrate is incorporated into a longer unstructured substrate, a K_d between 3 and 5 nM is observed. This result indicates that additional nonspecific contacts with the substrate substantially increase the affinity of binding of ClpA to the polypeptide. Equally, ClpA binds to αS1casein, an intrinsically unstructured protein,[16] with a K_d of ∼30 nM, which is ∼10-fold weaker than that of a 50-amino acid unstructured polypeptide substrate containing SsrA. This result indicates that ClpA does exhibit modest substrate specificity for the SsrA tag incorporated in an unstructured protein.

MATERIALS AND METHODS

Reagents and Buffers. All chemicals were reagent grade. All buffers were prepared with distilled and deionized water, which is produced from a Purelab Ultra Genetic system (Siemens Water Technology). Buffer H300 consists of 25 mM HEPES (pH 7.5) at 25 °C, 300 mM NaCl, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 10% (v/v) glycerol, where the 300 indicates the NaCl concentration.

Plasmids, Proteins, and Peptides. *E. coli* ClpA was purified as described previously.[17] The α-casein was purchased from Sigma (St. Louis, MO). This Sigma α-casein is a mixture of αS1 and αS2casein.[18] The gene encoding Titin I27 was kindly provided by A. Oberhauser (University of Texas Medical Branch, Galveston, TX).[19] The gene was subsequently cloned into an ssrA-containing pET30a plasmid. Titin I27-SsrA was overexpressed in *E. coli* strain JK10 (clpP::cat, Delta-lon, slyD::kan, LambdaDE3)[20] kindly provided by R. Sauer (Massachusetts Institute of Technology, Cambridge, MA). Polypeptides with lengths ranging from 11 to 50 amino acids were synthesized by CPC Scientific, Inc. (Sunnyvale, CA). All peptides were certified >95% pure based on reverse phase high-performance liquid chromatography, and the mass was confirmed by mass spectrometry. Fluorescence modification was accomplished by dialyzing the polypeptide substrate into 20 mM potassium phosphate buffer (pH 6.5 at 25 °C), 150 mM NaCl, 10 mM EDTA, and 1 mM TCEP. The polypeptide substrate was mixed with a 10-fold molar excess of fluorescein-5-maleimide from Invitrogen (Eugene, OR) and allowed to incubate for 4 h while being constantly shaken in the dark at ambient temperature. Excess dye was removed by dialysis, and then the peptide was passed over a HiPrep 26/10 desalting column from GE Healthcare (Piscataway, NJ) equilibrated with buffer H300. The dye:peptide ratio was determined to be in the range of 0.7−0.95.

The DNA fragment encoding αS1casein was obtained from *Nde*I- and *Eco*RI-digested recombinant plasmid pET17b-αS1casein, which was kindly provided by R. Valenta (Medical University of Vienna, Vienna, Austria). Recombinant plasmid pET28a(+)-αS1casein was generated by subcloning the DNA fragment of αS1casein between the *Nde*I and *Eco*RI sites of expression vector pET28a(+) (Novagen). Mutants and deletion variants were constructed by using standard polymerase chain reaction methods with the QuikChange II XL site-directed mutagenesis kit from Stratagene (Santa Clara, CA). A single cysteine was introduced into the N-terminus of αS1casein. STOP codons were inserted into different positions to generate the αS1casein truncations. All mutations were confirmed by DNA sequence analysis.

The DNA constructs of αS1casein truncations were transformed into *E. coli* strain BL21(DE3) (Novagen) and overexpressed after induction with 0.5 mM isopropyl β-D-thiogalactoside (IPTG). The recombinant proteins accumulated in the insoluble fraction. Cell paste from a 6 L growth was suspended in 200 mL of resuspension buffer [20 mM Tris (pH 8.0) at 4 °C]. Cells were then disrupted via sonication on ice. After sonication, cells were centrifuged at 10500 rpm in an SLA-3000 rotor at 4 °C for 30 min. The cell pellet was suspended in 100 mL of isolation buffer [2 M urea, 20 mM Tris, 0.5 M NaCl, and 2% Triton X-100 (pH 8.0) at 4 °C] and subjected to sonication. After disruption by sonication, cells were centrifuged at 10500 rpm in an SLA-3000 rotor at 4 °C for 30 min. The pellet was washed with 100 mL of isolation buffer as described above. Then the cell pellet was suspended in 100 mL of solubilization buffer [6 M guanidinium HCl, 20 mM Tris, 0.5 M NaCl, 5 mM imidazole, and 1 mM 2-mercaptoethanol (pH 8.0) at 4 °C] while being stirred for 30−60 min at ambient temperature. The sample was centrifuged at 10500 rpm in an SLA-3000 rotor at 4 °C for 60 min. The sample was loaded on a HisTrap FF column from GE Healthcare for further purification, after the sample had passed through a 0.45 μm filter. After the HisTrap FF column had been washed with 20−30 column volumes of wash buffer [6 M urea, 20 mM Tris, 0.5 M NaCl, 20 mM imidazole, and 1 mM 2-mercaptoethanol (pH 8.0) at 4 °C], αS1casein truncations were eluted with elution buffer [6 M urea, 20

Table 1. Polypeptide Substrates

name	no. of amino acids	sequence or source
N-Cys-50-SsrA	50	CLILHNKQLGMTGEVSFQAANTKSAANLKVKELRSKKKL**AANDENYALAA**
N-Cys-50	50	CEIIEDGKKHILILHNKQLGMYTGEVSFQAANTKSAANLKVKELRSKKKL
30-SsrA	30	KTKSAANLKVKELRSKKKL**AANDENYALAA**
SsrA	11	**AANDENYALAA**
αS1casein-127	127	C-terminal 127 amino acids of αs1casein
αS1casein-152	152	C-terminal 152 amino acids of αs1casein
αS1casein-177	177	C-terminal 177 amino acids of αs1casein

mM Tris, 0.5 M NaCl, 0.5 M imidazole, and 1 mM 2-mercaptoethanol (pH 8.0) at 4 °C]. Purified αS1casein truncations were dialyzed into 25 mM HEPES buffer (pH 7.5 at 25 °C), 1 M urea, 300 mM NaCl, and 1 mM TCEP and labeled with fluorescein-5-maleimide as described above. After dialysis into thrombin cleavage buffer [20 mM Tris (pH 8.4) at 25 °C, 1 M urea, 150 mM NaCl, 2.5 mM $CaCl_2$, and 1 mM 2-mercaptoethanol], the fluorescein-labeled αS1casein truncations were incubated with thrombin overnight and loaded onto a HisTrap FF column from GE Healthcare to separate the six-histidine tag and undigested protein from digested protein. The majority of the protein with the six-histidine tag removed eluted with wash buffer containing 20 mM imidazole. The eluted proteins were further purified through a HiLoad 16/60 Superdex 75 pg column from GE Healthcare with 20 mM Tris (pH 8.0 at 4 °C), 6 M urea, and 150 mM NaCl. After dialysis into H200 buffer, fluorescein-labeled αS1casein truncations were stored at −30 °C. The purity of the isolated protein was assessed to be >95% by sodium dodecyl sulfate−polyacrylamide gel electrophoresis with Coomasie brilliant blue staining.

Fluorescence Stopped-Flow Experiments. Single-turnover fluorescence stopped-flow experiments for examining ClpA-catalyzed polypeptide translocation were performed using an SX.20 Applied Photophysics (Leatherhead, U.K.) stopped-flow fluorometer as illustrated in Figure 1A and as previously described.[14,15] Experiments were conducted in buffer H300 at 25 °C, and the concentrations of reactants are shown in Figure 1A.

Single-turnover polypeptide translocation experiments were performed with N-Cys-50-SsrA and N-Cys-50 (see Table 1). As illustrated in Figure 1A, 1 μM ClpA monomer is mixed with 100 nM fluorescently modified polypeptide substrate and 150 μM ATPγS and loaded into one syringe of the stopped-flow fluorometer. The ATPγS is included to promote hexamer, which is required for ClpA to have polypeptide binding activity and therefore bind the SsrA-tagged substrate.[21,22] When ClpA is bound to the fluorescently modified polypeptide substrate, the fluorescence from the fluorescein is quenched.[14] Thus, the syringe contains ClpA assembled into an active oligomeric form that is statically prebound to the fluorescently modified peptide substrate poised to initiate translocation upon rapid mixing.

The second syringe contains 10 mM ATP and 200 μM SsrA peptide that is not labeled with a fluorophore. The two reactants illustrated in Figure 1A are rapidly mixed together; the fluorescein dye is excited at a λ_{ex} of 494 nm, and fluorescence emission is observed above 515 nm with a 515 nm long pass filter. When the contents of the two syringes are mixed, the 200 μM SsrA peptide (final mixing concentration of 100 μM) serves as a trap for unbound ClpA, where any unbound ClpA or any ClpA that dissociates from the polypeptide substrate during translocation will rapidly bind the trap. Thus, the trap inhibits ClpA from rebinding the fluorescently modified substrate and catalyzing a second round of translocation, thereby maintaining single-turnover conditions.

Anisotropy Titration Experiments. Steady-state anisotropy titrations were performed by titrating the fluorescently modified peptide with ClpA in buffer H300. Both samples were in the presence of 1 mM ATPγS to ensure that the concentration of the nucleotide remained constant throughout the titration. The incubation time for allowing each titration point to reach equilibrium was determined by fluorescence stopped-flow experiments for each substrate, which was found to be ∼30 min. Anisotropy signal changes were monitored by exciting fluorescein at a λ_{ex} of 494 nm and observing emission at a λ_{em} of 515 nm with a Fluorolog-3 spectrofluorometer (HORIBA Jobin Yvon).

ATPγS is slowly hydrolyzed by ClpA. The k_{cat} for ATPγS hydrolysis that we have determined is $(51 \pm 4) \times 10^{-3}$ min^{-1} (J. Miller, manuscript in preparation). Each titration point included incubation for ∼30 min. Using these numbers, we can calculate that only 20 μM of the 1 mM ATPγS is hydrolyzed over the entire time required for the titration, which is 2% of the total ATPγS present.

Competition steady-state anisotropy titrations were performed by titrating 54.9 nM fluorescently modified 50-amino acid polypeptide [N-Cys-50-SsrA (Table 1)] in the presence of varying concentrations of polypeptide substrate lacking a fluorophore with a solution of ClpA also containing 54.9 nM fluorescently modified polypeptide substrate. The fluorescently modified substrate was included in both solutions to ensure that a fixed concentration of the fluorescently modified substrate was maintained throughout the titration. Similarly, 1 mM ATPγS was also included in both the cuvette and the titrant to ensure a constant total concentration throughout the titration.

Fluorescence anisotropy measurements were performed with an L-format excitation and emission arrangement.[23] The anisotropy (r) is calculated according to eq 1,[24]

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}) \quad (1)$$

where I represents the detected emission intensity through a polarizer oriented in the vertical, V, or horizontal, H, direction and the first subscript indicates the orientation of the excitation polarizer and the second the emission polarizer. G is the G-factor, which is calculated as $G = I_{HV}/I_{HH}$. The G-factor represents the ratio of the sensitivities of the detection system for vertically and horizontally polarized light. By including the G-factor in the calculation of anisotropy, we correct the different transmission efficiencies due to the rotation of the emission polarizer.

The observed anisotropy can be affected by changes in quantum yield upon binding. If changes in quantum yield are

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present, then a change in total fluorescence, F_t, upon binding will be observed, where the total fluorescence is given by eq 2.[23]

$$F_t = I_{VV} + 2GI_{VH} \quad (2)$$

The total fluorescence was determined for each substrate used in this study at saturating concentrations of ClpA. In all cases, the total fluorescence, F_t, changed by less than 10% relative to that of the unbound substrate, which does not warrant correction of the anisotropy data.[25] Further, applying the correction factor described by Eftink[25] and subsequently analyzing the data did not significantly change the determined parameters.

The observed anisotropy (r_{obs}) at a given total ClpA concentration has contributions from both free fluorescently modified peptide, $[peptide]_f$, and bound fluorescently modified peptide, $[peptide]_b$, as given by eq 3,

$$r_{obs} = r_f\frac{[peptide]_f}{[peptide]_T} + r_b\frac{[peptide]_b}{[peptide]_T} \quad (3)$$

where r_f is the anisotropy of the free fluorescently modified peptide, r_b is the anisotropy of the bound complex, $[peptide]_f$ is the concentration of the free fluorescently modified peptide, $[peptide]_b$ is the concentration of the bound fluorescently modified peptide, and $[peptide]_T$ is the total concentration of the fluorescently modified peptide.

Because $[peptide]_T$ is the summation of $[peptide]_f$ and $[peptide]_b$, the relative anisotropy increase (Δr) can be described by eq 4.

$$\Delta r = \frac{r_{obs} - r_0}{r_0} = \frac{r_{obs} - r_f}{r_f} = \left(\frac{r_b - r_f}{r_f}\right)\frac{[peptide]_b}{[peptide]_T} = A\frac{[peptide]_b}{[peptide]_T} \quad (4)$$

where r_0 is the initial anisotropy, which is equal to r_f, and A is the amplitude, which is a function of r_b and r_f.

At any total concentration of fluorescently modified peptide $[peptide]_T$, Δr depends on the free ClpA hexamer concentration, $[ClpA_6]_f$, and the binding association equilibrium constant for ClpA hexamers binding to the fluorescently modified peptide (K_6), where K_6 is defined by the equilibrium in eq 5 and $K_{d,6} = 1/K_6$.

$$ClpA_6 + peptide \overset{K_6}{\rightleftharpoons} ClpA_6\text{-peptide} \quad (5)$$

Because ClpA hexamers reside in a dynamic equilibrium with monomers,[17,22,26] the concentration of ClpA hexamers is given by the equilibrium in eq 6,

$$6\{ClpA\} \overset{L_{6,app}}{\rightleftharpoons} \{ClpA_6\} \quad (6)$$

where $\{ClpA_6\}$ and $\{ClpA\}$ represent the summation of all of the nucleotide ligation states of hexameric and monomeric ClpA, respectively, and $L_{6,app}$ is given by eq 7,

$$L_{6,app} = \frac{\{ClpA_6\}}{\{ClpA\}^6} = L_{6,0}\frac{P_6}{P_1^6} \quad (7)$$

where P_1 is the partition function for all of the monomeric nucleotide ligation states normalized to the unligated monomer, P_6 is the partition function for all of the hexameric nucleotide ligation states normalized to the unligated hexamer, and $L_{6,0}$ is defined by the equilibrium given by eq 8, which represents hexamer formation in the absence of nucleotide, where the zero in the subscript represents no nucleotide bound.

$$6ClpA \overset{L_{6,0}}{\rightleftharpoons} ClpA_6 \quad (8)$$

Although it has been concluded that hexamers are not formed in the absence of nucleotide binding, the lack of observation of hexameric ClpA in the absence of nucleotide can also be described by a weak hexamerization equilibrium constant, $L_{6,0}$, relative to the equilibrium constant for dimerization ($L_{2,0} = 1 \times 10^4\ M^{-1}$) and tetramerization ($L_{4,0} = 2.4 \times 10^{14}\ M^{-3}$). We have estimated that the lack of observation of hexamers in our experiments performed in the absence of nucleotide[17,26] could be described by a hexamerization equilibrium constant with an upper limit of $\sim 1 \times 10^{24}\ M^{-5}$ based on simulations of the species fractions (simulations not shown).

We define the polypeptide binding constant K_6 by the equilibrium in eq 5. Incorporating K_6 and $L_{6,app}$ into eq 4 yields eq 9.

$$\Delta r = A\frac{[peptide]_b}{[peptide]_T} = A\frac{[ClpA_6\text{-peptide}]}{[peptide]_f + [ClpA_6\text{-peptide}]}$$

$$\Delta r = A\frac{K_6\{ClpA_6\}_f}{1 + K_6\{ClpA_6\}_f} = A\frac{K_6L_{6,app}\{ClpA\}_f^6}{1 + K_6L_{6,app}\{ClpA\}_f^6} \quad (9)$$

where $\{ClpA_6\}_f$ and $\{ClpA\}_f$ represent the concentrations of hexamers and the concentration of monomers, respectively, regardless of nucleotide ligation state but free of bound polypeptide, denoted by the subscript f. Because the independent variable in the NLLS analysis of the titration curves is the total ClpA concentration, the free monomer and hexamer concentrations are determined using the conservation of mass equation given by eq 10

$$[ClpA]_T = [ClpA]_f + 2L_{2,app}[ClpA]_f^2 + 4L_{4,app}[ClpA]_f^4 + 6L_{6,app}[ClpA]_f^6 + \bar{X}[peptide]_T \quad (10)$$

where $L_{2,app}$, $L_{4,app}$, and $L_{6,app}$ are given by eqs 11, 12, and 7, respectively

$$L_{2,app} = \frac{\{ClpA_2\}}{\{ClpA\}^2} = L_{2,0}\frac{P_2}{P_1^2} \quad (11)$$

$$L_{4,app} = \frac{\{ClpA_4\}}{\{ClpA\}^4} = L_{4,0}\frac{P_4}{P_1^4} \quad (12)$$

and the extent of binding, $\bar{X}$, is given by eq 13.

$$\bar{X} = \frac{[ClpA]_b}{[peptide]_T} = \frac{6[ClpA_6\text{-peptide}]}{[peptide]_T} = \frac{6K_6L_{6,app}[ClpA]_f^6}{1 + K_6L_{6,app}[ClpA]_f^6} \quad (13)$$

where $[ClpA]_b$ is the concentration of peptide-bound ClpA monomers. $ClpA_6$-peptide represents the complex of the ClpA hexamer bound to the fluorescently modified polypeptide.

For αS1casein substrates that exhibited more than one hexamer bound, the extent of binding is given by eq 14,

$$\bar{X} = (6K_{61}L_{6,app}[ClpA]_f^6 + 12K_{61}K_{62}L_{6,app}[ClpA]_f^{12}) / (1 + K_{61}L_{6,app}[ClpA]_f^6 + K_{61}K_{62}L_{6,app}[ClpA]_f^{12}) \quad (14)$$

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where K_{61} and K_{62} represent the binding constants for the first and second hexamer, respectively.

The relationship between the relative anisotropy increase and the extent of binding, $\bar{X}$, was found to be nonlinear (see Figure 3B, for example). Thus, the titration curves were analyzed using the empirical function approach as previously described.[27−29] In this approach, the relationship between the relative anisotropy increase, Δr, and the extent of binding, $\bar{X}$, is described by an empirical function based on the shape of the relative anisotropy increase versus the extent of binding. In this study, the relationship was described well by a second-degree polynomial given by eq 15,

$$\Delta r = a + b\bar{X} + c\bar{X}^2 \quad (15)$$

where a, b, and c represent parameters.

Sets of four titration curves were subjected to global NLLS analysis by implicitly solving the system of equations given by eqs 10, 13, and 15 using Scientist (Micromath, St. Louis, MO). In this analysis, K_6, $L_{2,app}$, $L_{4,app}$, and $L_{6,app}$ and parameters a, b, and c are all global parameters. In our previous examination of ClpA self-association in the absence of nucleotide, we did not detect dimers at 25 °C, but dimers were detected at ∼4 and 37 °C. Thus, we approximated $L_{2,0} = 1 \times 10^4$ M^{-1} and determined $L_{4,0}$ to be 2.4×10^{14} M^{-3}.[17,26] In sedimentation velocity experiments performed at 25 °C in the presence of 1 mM ATPγS, we determined $L_{4,app} = (3 \pm 2) \times 10^{20}$ M^{-3} and $L_{6,app} = (5 \pm 1) \times 10^{34}$ M^{-5} but did not detect dimers, leading us to conclude that $L_{2,app}$ is no larger than $L_{2,0}$ (J. Lin, manuscript in preparation). All of the anisotropy titrations of the synthetic polypeptide substrates with parameters listed in Table 2 were subjected to NLLS by constraining $L_{2,app}$ to 1×10^4 M^{-1}, $L_{4,app}$ to 3×10^{20} M^{-3}, and $L_{6,app}$ to 5×10^{34} M^{-5} in eq 10, and values of $K_{d,6}$ that were within error of those listed in Table 2 were determined. The values of $L_{2,app}$, $L_{4,app}$, and $L_{6,app}$ were also allowed to float as fitting parameters, and $L_{2,app}$ and $L_{4,app}$ converged to insignificant values relative to the term that describes the free hexamer concentration, i.e., $6L_{6,app}[\text{ClpA}]_f^6$. However, $L_{6,app}$ converged to a value of $(1.4 \pm 0.6) \times 10^{33}$ M^{-5}, which is similar to the value determined from independent sedimentation velocity experiments. Thus, we concluded that the parameter is well constrained in the fitting, and the values are listed in Table 2.

Table 2. Binding and Assembly Parameters for ClpA Binding Polypeptides

name	no. of amino acids	$K_{d,6}$ (nM)	$L_{6,app}$ (M^{-5})
N-Cys-50-SsrA	50	4.7 ± 0.8	$(1.4 \pm 0.6) \times 10^{33}$
N-Cys-50	50	30 ± 2	1.4×10^{33}[a]
30-SsrA	30	3 ± 1	1.4×10^{33}[a]
SsrA	11	200 ± 30	1.4×10^{33}[a]

[a]Data were collected with competition steady-state anisotropy titrations by inhibiting the binding of fluorescein-labeled N-Cys-50-SsrA (Flu-N-Cys-50-SsrA). $L_{6,app}$ was constrained to be the same as $L_{6,app}$ of N-Cys-50-SsrA.

Competition titrations were subjected to NLLS analysis using eqs 13 and 15−17. Equation 16 incorporates a term that accounts for binding to the competitor given by the extent of binding to the competitor, $\overline{X_C}$, times the total competitor concentration, $[\text{competitor}]_T$.

$$[\text{ClpA}]_T = [\text{ClpA}]_f + 2L_{2,app}[\text{ClpA}]_f^2 + 4L_{4,app}[\text{ClpA}]_f^4 + 6L_{6,app}[\text{ClpA}]_f^6 + \bar{X}[\text{peptide}]_T + \overline{X_C}[\text{competitor}]_T \quad (16)$$

The extent of binding, $\overline{X_C}$, to the competitor is given by eq 17.

$$\overline{X_C} = \frac{[\text{ClpA}]_b}{[\text{competitor}]_T} = \frac{6[\text{ClpA}_6\text{-competitor}]}{[\text{competitor}]_T} = \frac{6K_{6C}L_{6,app}[\text{ClpA}]_f^6}{1 + K_{6C}L_{6,app}[\text{ClpA}]_f^6} \quad (17)$$

K_{6C} is the binding equilibrium constant for the equilibrium given by eq 18 and $K_{d,6C} = 1/K_{6C}$.

$$\text{ClpA}_6 + \text{competitor} \overset{K_{6C}}{\rightleftharpoons} \text{ClpA}_6\text{-competitor} \quad (18)$$

In this analysis K_6 and $L_{6,app}$ were constrained to the value determined in Table 2 for N-Cys-50-SsrA. Thus, the only floating parameters in this analysis are K_{6C}, a, b, and c.

The titration data in Figure 4A were subjected to global NLLS analysis using eq 19

$$\bar{X} = \frac{K[\text{ClpA}_6]_f}{1 + K[\text{ClpA}_6]_f} \quad (19)$$

and the conservation of mass equation given by eq 20

$$\frac{[\text{ClpA}_1]_T}{6} = [\text{ClpA}_6]_T = [\text{ClpA}_6]_f + \bar{X}[\text{peptide}]_T \quad (20)$$

where $[\text{ClpA}_6]_T$ represents the total ClpA hexamer concentration given by the total ClpA monomer concentration divided by six and $[\text{ClpA}_6]_f$ represents the free ClpA hexamer concentration.

Binding Density Function Analysis. Each set of titrations collected at four different total polypeptide concentrations was subjected to binding density function (BDF) analysis as previously described.[29−32] This was done to determine the dependence of the relative anisotropy increase on the extent of binding ($[\text{ClpA}]_{\text{monomer-bound}}/[\text{peptide}]_T$) and the maximal stoichiometry at a saturating ClpA concentration. BDF analysis was accomplished by drawing a series of horizontal lines that intersect each titration curve. Each horizontal line represents a constant relative anisotropy increase and intersects each titration curve collected at a different total polypeptide concentration at one point. Because the relative anisotropy increase is the same at each point of intersection, the thermodynamic state of the macromolecule (fluorescently modified polypeptide substrate) must be the same even though a different total ligand concentration ($[\text{ClpA}]_T$) was required to achieve the observed signal change. If the thermodynamic state of the macromolecule is the same, then the extent of binding, $\bar{X}$, must be the same.[29−32] Because the extent of binding is governed by the free ligand concentration ($[\text{ClpA}]_f$), the free ClpA concentration at each point of intersection must also be the same. Thus, to determine the extent of binding, the total ClpA concentration at each point of intersection was plotted as a function of the total polypeptide concentration and subjected to NLLS analysis using the conservation of mass equation given by eq 21,

$$[\text{ClpA}]_T = \bar{X}[\text{peptide}]_T + B \quad (21)$$

where $[\text{peptide}]_T$ represents the independent variable, $[\text{ClpA}]_T$ represents the dependent variable, the extent of binding ($\bar{X}$ =

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$[ClpA]_{monomer\text{-}bound}/[peptide]_T$), represents the slope, and B represents the y-intercept, which would be the sum of all of the terms containing $[ClpA]_f$ in eq 10 given by eq 22.

$$B = [ClpA]_f + 2L_{2,app}[ClpA]_f^2 + 4L_{4,app}[ClpA]_f^4 + 6L_{6,app}[ClpA]_f^6 \quad (22)$$

From this analysis, a value of the extent of binding ($[ClpA]_{monomer\text{-}bound}/[peptide]_T$) for each arbitrary horizontal line drawn across the four titration curves at a constant relative anisotropy increase is determined. A plot of the relative anisotropy increase as a function of the extent of binding ($[ClpA]_{monomer\text{-}bound}/[peptide]_T$) is constructed to determine the relationship between the signal change and the extent of binding and predict the maximal stoichiometry at saturating ClpA concentrations.

BDF analysis was also applied to the data plotted as anisotropy versus $[ClpA]_T$, and the conclusions are identical (see Figure 3A–H of the Supporting Information).

Circular Dichroism (CD). CD measurements were performed with a Jasco (Easton, MD) J-815 spectrometer in buffer H200, which contains 200 mM NaCl instead of 300 mM NaCl, which buffer H300 contains. The NaCl concentration was reduced to allow for collection of data below 215 nm.

RESULTS

Polypeptide Translocation. We previously examined ClpA-catalyzed polypeptide translocation in the absence of the proteolytic component, ClpP, using a series of synthetic polypeptide substrates ranging in length from 12 to 50 amino acids.[14] All of these substrates contained the 11-amino acid SsrA sequence at the carboxy terminus and a cysteine at the amino terminus (see Table 1). The sequences of these substrates are based on the sequence of the full-length Titin I27 domain that had been previously used to examine ClpXP-catalyzed protein degradation.[20]

Weber-Ban and co-workers reported that they were unable to form a stably prebound complex between ClpA and an SsrA-containing T4 lysozyme in the presence of 1 mM ATPγS.[6] In contrast, they could form a complex with an SsrA-containing λ repressor protein. They hypothesized that this was because T4 lysozyme forms a stably folded structure but λ repressor fluctuates rapidly between folded and unfolded states. In support of this hypothesis, it is known that ClpAP binds and degrades unstructured polypeptide substrates that do not contain any known amino acid binding sequence for ClpA.[9] From these observations, we hypothesize that the short polypeptide substrates used in our previous study[14] may not contain much structure despite the fact that these substrates are truncations of Titin I27, a highly structured and stable protein.[19,33] Because these relatively short polypeptide substrates may not be highly structured, we asked the question of whether ClpA will bind and translocate an otherwise identical substrate without the SsrA sequence.

To answer this question, single-turnover fluorescence stopped-flow experiments were performed as described previously.[14,34] Substrate translocation was examined using two 50-amino acid substrates, one containing the SsrA sequence and one with the same length of 50 amino acids without the SsrA sequence. The experiments were performed by mixing 1 μM ClpA monomer with 100 nM fluorescently modified polypeptide substrate and 150 μM ATPγS and loading this solution into one syringe of the stopped-flow instrument (see Figure 1A). Into the other syringe were loaded

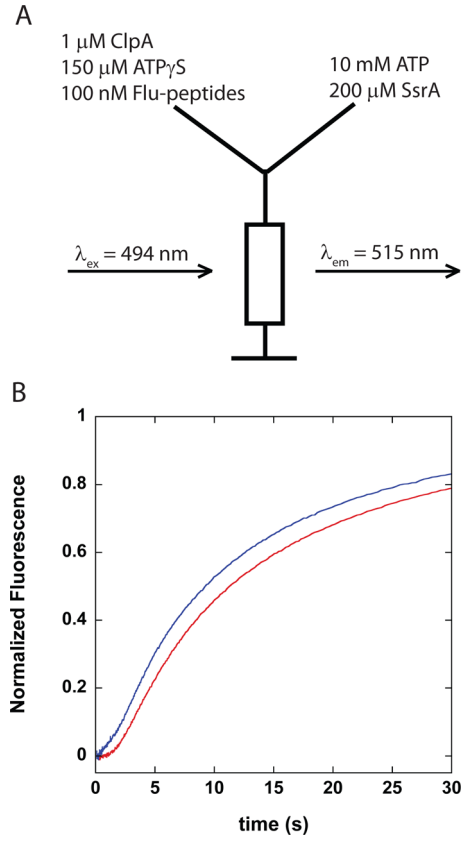

Figure 1. Single-turnover fluorescence stopped-flow experimental design for ClpA translocation on fluorescein-labeled polypeptides. (A) Experimental scheme illustrating the contents of each syringe that will be rapidly mixed together and excited at a λ_{ex} of 494 nm and emission observed at a λ_{em} of 515 nm. (B) Kinetic time courses for ClpA-catalyzed polypeptide translocation on Flu-N-Cys-50-SsrA (red) and Flu-N-Cys-50 (blue) (see Table 1) in buffer H300 at 25 °C.

10 mM ATP and 200 μM unmodified SsrA polypeptide to serve as a protein trap (see Materials and Methods). The two substrates were rapidly mixed, and fluorescence from the fluorescein at the amino terminus was monitored. The time courses for the two substrates are similar but not identical (Figure 1B). The time course for the 50-amino acid substrate lacking SsrA exhibits a lag that is not as flat as the lag exhibited by the 50-amino acid substrate containing SsrA. We have shown through simulations that a "sloped" lag indicates that dissociation during translocation is significant.[34] This result may indicate that a faster dissociation rate constant is apparent on a substrate with SsrA removed. Also, it is clear that ClpA binds to the substrate lacking SsrA, but it is unclear where in the primary structure ClpA binds the substrate. However, because of the lag, the time course is consistent with translocation.

Secondary Structure of Synthetic Polypeptide Substrates. Our observation that ClpA binds and translocates a polypeptide substrate lacking any known binding sequence suggests that the short polypeptide substrates used here lack significant structure. To test this, we collected CD spectra for the 30- and 50-amino acid polypeptide substrates as well as α-casein, a known intrinsically unstructured protein and natural

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substrate for ClpAP.[35,36] In addition, we collected a CD spectrum for the Titin I27 domain-containing SsrA at the carboxy terminus, because the short polypeptide substrates represent truncations of this protein.

Figure 2 shows the CD spectra for Titin I27, α-casein, and the 30- and 50-amino acid polypeptide substrates. As expected,

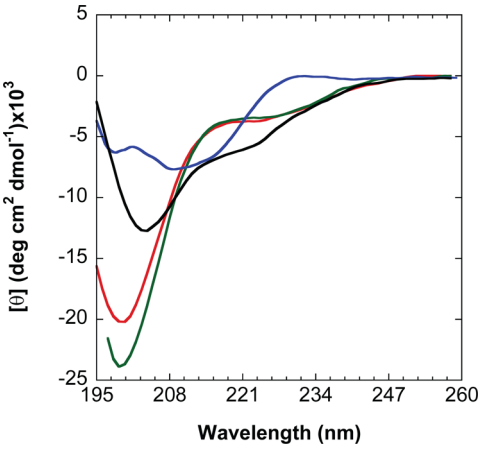

Figure 2. CD spectra for different polypeptide substrates at 25 °C: (green) 30-SsrA, (red) N-Cys-50-SsrA, (blue) Titin I27-SsrA, and (black) α-casein.

Titin I27-SsrA exhibits a CD spectrum consistent with a primarily β-sheet protein consistent with previously published CD spectra of this protein.[20,37] The α-casein also exhibits a CD spectrum consistent with a random coil as reported by others.[16] Although the 30- and 50-amino acid polypeptide substrates represent truncations of Titin I27, the CD spectra do not indicate the presence of significant amounts of β-sheet or helical structure. In addition, the large negative molar ellipticity between ~200 and ~205 nm for both α-casein and the 30- and 50-amino acid substrates is consistent with a mostly random coil structure.[38] Thus, we conclude from the CD spectra that the short synthetic polypeptide substrates in Table 1 do not exhibit significant structure.

Substrate Specificity for SsrA. The fluorescence stopped-flow experiments suggest that ClpA binds and translocates the 50-amino acid substrate regardless of whether SsrA is present. CD measurements indicate that the substrate is unstructured. Therefore, one natural question is whether ClpA exhibits substrate specificity for the SsrA sequence relative to the substrate lacking SsrA when both polypeptides are unstructured. To address this question, we performed anisotropy titrations as described in Materials and Methods. Briefly, anisotropy titrations were performed with the fluorescently modified 50-amino acid substrate containing the SsrA sequence at the carboxy terminus and fluorescein attached to the cysteine at the amino terminus (N-Cys-50-SsrA in Table 1). ClpA with 1 mM ATPγS was titrated into a cuvette containing 54.9, 110, 165, and 220 nM polypeptide substrate and 1 mM ATPγS. ATPγS was used because it induces hexamer formation more efficiently than any other nucleoside triphosphate other than hydrolyzable ATP.[21,22] Figure 3A shows the set of four anisotropy titrations plotted as the relative anisotropy increase (see eq 4) as a function of the total ClpA concentration in monomer units (for reference, the same data are plotted as anisotropy vs $[ClpA]_T$ in Figure 3A of the Supporting Information).

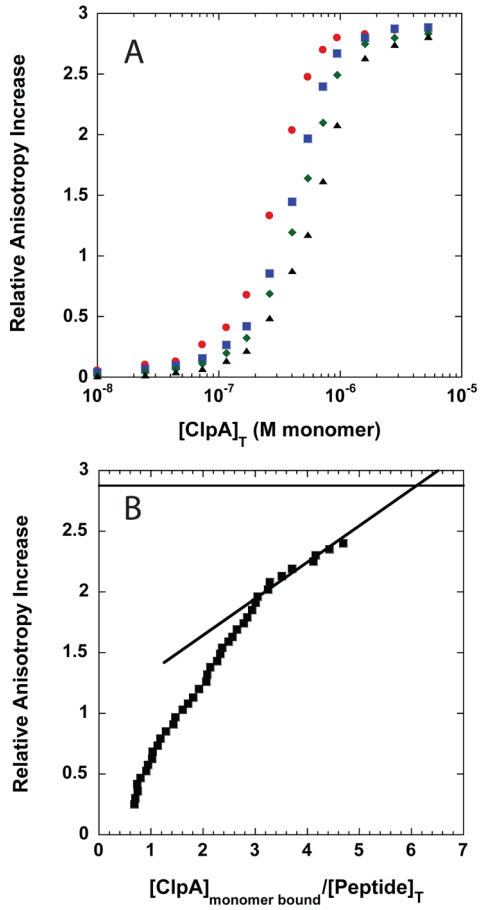

Figure 3. Steady-state anisotropy titrations of Flu-N-Cys-50-SsrA (see Table 1) with ClpA in the presence of 1 mM ATPγS in buffer H300 at 25 °C. (A) Relative anisotropy increase for titrations with 54.9 nM (red circles), 110 nM (blue squares), 165 nM (green diamonds), and 220 nM (black triangles) Flu-N-Cys-50-SsrA titrated with ClpA. (B) Relative anisotropy increase as a function of the extent of binding ($[ClpA]_{monomer\text{-}bound}/[peptide]_T$) determined from binding density function (BDF) analysis of the titrations presented in panel A (see Materials and Methods). The solid horizontal line represents the maximal observed increase in the magnitude of the signal for the four titrations in panel A of ~2.85. The sloped solid line represents an extrapolation from the linear phase between relative anisotropy increases of ~2 and ~2.5. The extrapolation intersects the horizontal line (maximal change in the magnitude of the signal) at ~6 ClpA monomers bound per polypeptide substrate.

Strikingly, all four titration curves reach saturation in ~1 log unit of ClpA concentration. This observation is not consistent with 1:1 binding, where all of the ClpA is hexameric and only one hexamer binds per polypeptide substrate because such a binding isotherm would be expected to saturate in ~2 log units. Rather, it is consistent with ClpA changing its assembly state during the course of the titration.

In our previous sedimentation velocity experiments, we observed one hexamer bound to this substrate at 2.75 μM polypeptide substrate, 8.5 μM ClpA monomer, and 1 mM ATPγS.[22] Moreover, we showed that ClpA, in the presence of 1 mM ATPγS, was in a mixture of monomers, hexamers, and potentially intermediate oligomers. Thus, the observation of cooperativity in these titrations could mean that polypeptide substrate induces hexamer formation, an observation that has been made for other AAA+ motors,[39] or that multiple oligomers bind to the polypeptide substrate. If the former is

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true, the observation is consistent with ClpA residing in a dynamic equilibrium of monomers and hexamers.

To determine the relationship between the observed signal change and the extent of binding ($[ClpA]_{monomer\text{-}bound}/[peptide]_T$), the set of four titrations were subjected to BDF[30−32] analysis (see Materials and Methods and Figure 3B). From this analysis, a determination of the extent of binding at several values of the relative anisotropy increase was determined and is plotted in Figure 3B. In the analysis presented here, the polypeptide substrate is considered the macromolecule and ClpA is considered as the ligand. This analysis requires a nondissociating macromolecule. However, the analysis still applies if the ligand changes its assembly state, in this case ClpA.[40]

BDF analysis shows that the relationship between the relative anisotropy increase and the extent of binding is nonlinear. Figure 3B shows that the relative anisotropy increase increases linearly to a value of ∼2, followed by a second linear phase between ∼2 and 2.5. A short extrapolation of the second linear phase to the maximal observed relative anisotropy increase of ∼2.85, i.e., saturation point in Figure 3A, indicates that the maximal signal represents, on average, six monomers bound per polypeptide substrate, consistent with one hexamer bound per peptide at saturation (see Figure 3B).

The observation of six monomers bound per polypeptide at saturation is consistent with our previous sedimentation velocity experiments that showed only hexamers bound to the fluorescently modified polypeptide substrate in the presence of 1 mM ATPγS.[22] Moreover, Piszczek and co-workers reported no signal change in ITC experiments upon titration of ClpA in the absence of nucleotide with the SsrA peptide,[13] indicating that monomers and tetramers do not bind to the polypeptide in the absence of a nucleotide. Consistently, we reported a similar observation from fluorescence stopped-flow experiments, where no signal change indicating binding was observed upon mixing fluorescently modified polypeptide substrate with ClpA in the absence of nucleotide.[14] However, none of these observations rule out the possibility that small populations of nucleotide-bound monomers and tetramers could interact with the polypeptide substrate.

As has been done by others upon examination of binding of ClpA to SsrA polypeptide,[13] and because our data support one hexamer binding one substrate, we subjected the data to analysis using a 1:1 binding model given by eqs 15, 19, and 20. In this analysis, it is assumed that all of the ClpA is in the hexameric state by dividing the total monomer concentration by 6 (see eq 20). The results of the analysis are shown in Figure 4A, and the data are not described well by such a model. This is likely because the titration curves are too steep to be described by a 1:1 binding model. This further indicates that not all of the ClpA in solution is in the hexameric state.

The data in Figure 3A were then subjected to global NLLS analysis by simultaneously solving eqs 10, 13, and 15 that account for the distribution of oligomers in solution. However, it assumes that only hexameric ClpA is competent for binding the polypeptide substrate. This model clearly describes all four isotherms (see Figure 4B). Furthermore, the fit was not improved upon incorporation of terms that would account for monomers, dimers, or tetramers binding to the polypeptide into the extent of binding equation (eq 13). In fact, the monomer, dimer, and tetramer peptide binding constants (K_1, K_2, and K_4, respectively) all floated to insignificant values. This observation indicates that if oligomers smaller than hexamers

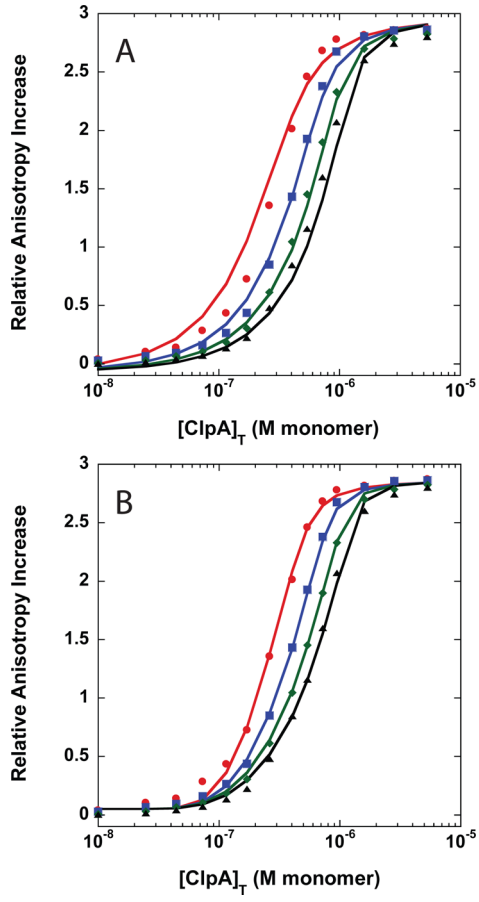

Figure 4. Global NLLS analysis of steady-state anisotropy titration data collected at 54.9 nM (red circles), 110 nM (blue squares), 165 nM (green diamonds), and 220 nM (black triangles) Flu-N-Cys-50-SsrA titrated with ClpA examined with (A) a 1:1 binding model (eqs 15, 19, and 20) or (B) a hexamer binding model incorporating linkage to macromolecular assembly (eqs 10, 13, and 15). The solid lines represent the best fits with a K_6 of $(2.1 \pm 0.3) \times 10^8$ M^{-1} ($K_{d,6}$ = 4.7 ± 0.8 nM) and an $L_{6,app}$ of $(1.4 \pm 0.6) \times 10^{33}$ M^{-5} (see Table 2).

interact with the polypeptide the concentration of the bound species is below the detection limit of this analysis.

Interestingly, from the analysis presented in Figure 4B, the affinity for the 50-amino acid substrate containing the 11-amino acid SsrA sequence at the carboxy terminus is as follows: K_6 = $(2.1 \pm 0.3) \times 10^8$ M^{-1} ($K_{d,6}$ = 4.7 ± 0.8 nM) and $L_{6,app}$ = $(1.4 \pm 0.6) \times 10^{33}$ M^{-5} (see Table 2) (where the parameters are defined in eqs 5 and 6). The $K_{d,6}$ value of 4.7 ± 0.8 nM is surprising because previous reports of binding to the 11-amino acid SsrA sequence reported a binding constant of ∼100 nM, which is ∼20-fold weaker.[13] This could indicate that ClpA binds tighter to the fluorescent dye at the amino terminus than to the SsrA sequence at the carboxy terminus.

To determine if ClpA exhibits specific interactions with the fluorescent modification, we performed competition titrations. These experiments were conducted by titrating 54.9 nM fluorescein-modified 50-amino acid substrate in the presence of 50, 100, 150, and 200 nM 50-amino acid substrate with no fluorophore. The four titration curves were subjected to global NLLS analysis using eqs 13 and 15−17. (see Figure 1A of the Supporting Information). From this analysis, a K_{6c} of $(2.6 \pm 0.2) \times 10^8$ M^{-1} ($K_{d,6c}$ = 3.9 ± 0.4 nM) was determined and an $L_{6,app}$ of $(1.4 \pm 0.6) \times 10^{33}$ M^{-5} was constrained. The presence

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of the dye does not substantially affect the affinity because the fluorescently modified substrate exhibits a $K_{d,6}$ of 4.7 ± 0.8 nM and the unmodified substrate exhibits a $K_{d,6c}$ of 3.9 ± 0.4 nM, which are within error of each other.

Competition titrations were also performed with a 30-amino acid substrate containing SsrA at the carboxy terminus, but lacking the dye (30-SsrA in Table 1). The affinity for this substrate (K_{6c}) was found to be $(3 \pm 1) \times 10^8$ M^{-1} ($K_{d,6c}$ = 3 ± 1 nM) (see Table 2 and Figure 1B of the Supporting Information).

ClpA seems to bind ~30−50-fold tighter to longer unstructured substrates containing SsrA compared to the previously reported affinity constant for only the 11-amino acid SsrA sequence, although the conditions are not identical. To test this under our solution conditions, we performed competition titrations with the SsrA sequence. Titrations were performed with 54.9 nM 50-amino acid substrate in the presence of 50, 200, 350, and 500 nM SsrA polypeptide (see Figure 1C of the Supporting Information). From this, we determined an affinity for SsrA (K_{6c}) of $(5.1 \pm 0.7) \times 10^6$ M^{-1} ($K_{d,6c}$ = 200 ± 30 nM), similar to the previously reported value of ~100 nM. This large difference in binding affinity between the SsrA sequence alone and the SsrA sequence present in longer substrates could indicate that the 11-amino acid SsrA sequence does not provide all of the necessary contacts for the formation of a stable complex and additional random sequence may provide the necessary stabilizing contacts.

The CD measurements in Figure 2 show that the 30- and 50-amino acid substrates are most consistent with a random coil structure. Wickner and co-workers showed that ClpAP would bind and degrade unfolded proteins containing no specific binding sequence for ClpA.[9] Our translocation experiments in Figure 1 show that ClpA will bind and translocate both a 50-amino acid substrate containing SsrA and an equivalent length substrate without SsrA. Thus, we asked the question of whether ClpA binds specifically to SsrA in these unstructured polypeptide substrates. To answer this question, we performed competition titrations with the 50-amino acid substrate lacking SsrA. This was done by titrating 54.9 nM fluorescently modified 50-amino acid substrate in the presence of 50, 200, 350, and 500 nM 50-amino acid substrate lacking SsrA (see Figure 1D of the Supporting Information). The affinity for the 50-amino acid substrate lacking SsrA (K_{6c}) was found to be $(3.4 \pm 0.3) \times 10^7$ M^{-1} ($K_{d,6c}$ = 30 ± 2 nM), which is ~10-fold weaker than that of the 50-amino acid substrate containing SsrA [K_{6c} = $(2.6 \pm 0.2) \times 10^8$ M^{-1} ($K_{d,6c}$ = 3.9 ± 0.4 nM)]. Thus, ClpA is ~10 times more specific for SsrA in an unfolded substrate than in an equivalent length substrate lacking SsrA.

We performed translocation experiments as illustrated in Figure 1 by prebinding ClpA in the presence of ATPγS to the Titin I27 protein containing the SsrA sequence at the carboxy terminus. However, the resultant time course did not indicate that ClpA was prebound (data not shown). A similar observation was made by Weber-Ban and co-workers with an SsrA-tagged T4 lysozyme; they concluded that ClpA in the presence of 1 mM ATPγS was unable to bind to SsrA when the sequence was juxtaposed with a stably folded protein.[6]

To determine if the lack of translocation observed with Titin I27 is due to the inability of ClpA to bind SsrA in Titin I27, we titrated Titin I27 containing a fluorescein at the amino terminus and SsrA at the carboxy terminus with ClpA (see Figure 5). Anisotropy titrations of Titin I27 with ClpA were performed at 50 and 150 nM Titin I27. The signal indicates binding does not

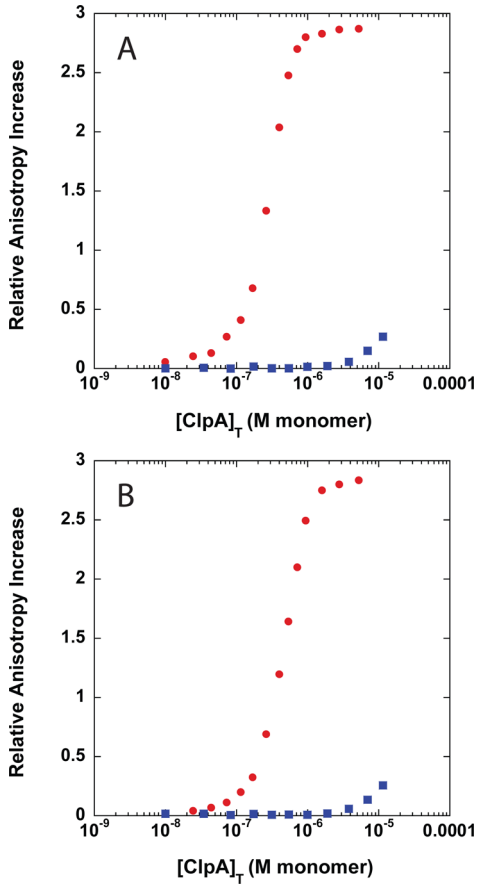

Figure 5. Binding isotherms from titrations of Flu-N-Cys-50-SsrA and Flu-Titin-I27-SsrA with ClpA in the presence of 1 mM ATPγS in buffer H300 at 25 °C monitoring steady-state anisotropy at 515 nm: (A) 54.9 nM Flu-N-Cys-50-SsrA (red circles) or 50 nM Flu-Titin-I27-SsrA (blue squares) titrated with ClpA and (B) 110 nM Flu-N-Cys-50-SsrA (red circles) or 150 nM Flu-Titin-I27-SsrA (blue squares) titrated with ClpA.

occur until ~4 μM ClpA is reached, and it starts to increase up to the limit of ClpA concentration that we can achieve in these experiments of ~10 μM [Figure 5A,B (blue squares)]. Clearly, binding to a substrate that has stable secondary structure flanking SsrA is substantially weaker than that to SsrA in a polypeptide substrate with a sequence similar to that of Titin I27. On the basis of the assembly equilibrium constants, we would predict the hexameric ClpA concentration to be ~1.4 μM at a total ClpA monomer concentration of 10 μM. Thus, from these titrations, we can conclude only that the apparent K_d for hexamers binding to Titin is >1.4 μM.

ClpA Binding to Long Unstructured Proteins. If ClpA is binding nonspecifically to unstructured proteins, then this suggests that multiple ClpA hexamers may bind to polypeptide chains of sufficient length to accommodate two hexamers. To determine if multiple hexamers can bind to unstructured substrates, we constructed a series of substrates from the intrinsically unstructured αS1casein protein, which is a natural substrate for ClpAP. Full-length αS1casein contains 214 amino acids. We constructed truncations of this protein with 127, 152, and 177 amino acids. Each substrate was engineered to contain a single cysteine at the amino terminus that has been fluorescently modified with fluorescein-5-maleimide. Each αS1casein construct was titrated with ClpA at 50, 100, 150, and 200 nM αS1casein and subjected to BDF analysis (see

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Figure 2 of the Supporting Information). Interestingly, only one hexamer was found to be bound to the 127-amino acid substrate. However, the 152- and 177-amino acid substrates were shown to have two hexamers bound per substrate (see Table 3). Further, αS1casein-127 exhibits a $K_{d,6,1}$ of 33 ± 2 nM,

Table 3. Binding and Assembly Parameters for Binding of ClpA to αS1Casein Truncations

name	no. of amino acids	$K_{d,6,1}$ (nM)	$K_{d,6,2}$ (nM)	$L_{6,app}$ (M^{-5})
αS1casein-127	127	33 ± 2	not available	(9 ± 8) × 10^{39}
αS1casein-152	152	18 ± 4	610 ± 150	(10 ± 3) × 10^{39}
αS1casein-177	177	11 ± 4	310 ± 50	(3.0 ± 0.7) × 10^{39}

which is comparable to the binding constant for binding the 50-amino acid substrate lacking SsrA ($K_{d,6}$ = 30 ± 2 nM). In contrast, the first hexamer binding site is tighter for both the 152- and 177-amino acid substrates (see Table 3). However, the second binding site is substantially weaker, likely as a consequence of steric interactions between the two hexamers.

DISCUSSION

Application of Anisotropy in Examining Binding of ClpA to Polypeptide Substrates. Anisotropy is sensitive to changes in the rotational correlation time of a fluorophore. Here we have shown that anisotropy measurements are a viable experimental approach to examining binding of ClpA to polypeptide substrates. To this end, we used synthetic polypeptide substrates ranging in length from the smallest 11-amino acid SsrA sequence to a 50-amino acid sequence. We also used substrates overexpressed and isolated from *E. coli* that ranged in length from 127 to 177 amino acids. All in all, the substrates used in this study have molecular masses ranging from 1.1 to ∼20 kDa. Because a hexamer of ClpA has a molecular mass of 505 kDa, the change in rotational correlation time upon binding is expected to be large. Consistently, we observe an ∼3-fold increase in anisotropy upon binding of ClpA to the 50-amino acid substrate.

The value of anisotropy can be affected by changes in total fluorescence upon binding. In other words, if ClpA affects the quantum yield of fluorescein upon binding of the labeled substrate, then the measured value of anisotropy will be affected.[25] In our studies, ClpA does not exhibit significant changes in quantum yield upon binding; i.e., little change in total fluorescence upon binding is observed. Thus, because the molecular mass of hexameric ClpA is 505 kDa and the substrate the enzyme binds is small, anisotropy is an ideal technique for examining ClpA binding. Moreover, the strategy presented here will be generally applicable for examining binding by other AAA + protein unfoldases, especially those that do not interact with proteases, and therefore, a K_m for proteolytic degradation cannot be determined, e.g., ClpB/Hsp104,[41,42] Katanin, and Spastin.[43]

Thermodynamic Linkage of Macromolecular Assembly to Polypeptide Binding. Most available knowledge regarding which substrates ClpA recognizes has been garnered through the observation of steady-state degradation catalyzed by the proteolytic component, ClpP, in the ClpAP complex.[44,45] Consequently, most of the quantitative information about relative affinities of binding of ClpA to tag sequences comes in the form of Michaelis constants, K_m.[10,11] Reported values of K_m range from 1.5 to 1.7 μM for SsrA-tagged substrates and ∼1.2 μM for α-casein.[10−12] Although K_m is a useful and important parameter to determine, it is not likely to be equal to the binding equilibrium constant for binding of ClpAP to the polypeptide substrate. This is because in a steady-state experiment the observation of proteolysis is limited by the slowest step in repeating cycles of ATP binding, polypeptide binding, translocation, product release, and potentially changes in the assembly state of ClpA, ClpP, and ClpAP.

In this study, the polypeptide at a fixed concentration is titrated with ClpA, with the total monomer concentration of ClpA ranging from 10 nM to 10 μM in the presence of a fixed ATPγS concentration of 1 mM. As shown in Figure 4A, the data are not adequately described if we assume that only hexamers of ClpA are in solution in the presence of 1 mM ATPγS and subject the data to analysis using a 1:1 binding model, i.e., one hexamer binding one polypeptide substrate. This observation indicates that ClpA resides in a dynamic equilibrium of hexamers and other oligomers, likely a monomer−hexamer equilibrium in the presence of 1 mM ATPγS. Therefore, to quantitatively analyze the set of four isotherms, we must incorporate a model for macromolecular assembly.

The formation of hexamers active in polypeptide binding requires either ATP binding or ATPγS binding.[22] Therefore, hexamer formation is thermodynamically linked to the nucleotide ligation state. To model the linkage of nucleotide binding to ClpA hexamer formation, there is a need for an accurate model for the self-association reaction of ClpA in the absence of nucleotide.

We have previously shown that ClpA resides in a monomer−dimer−tetramer equilibrium in the absence of nucleotide.[17,26] However, dimers are not significantly populated at 25 °C. This is described by the equilibrium given in eqs 23 and 24, where $L_{2,0}$ and $L_{4,0}$ represent the dimerization constant and the tetramerization constant, respectively, both in the absence of a nucleotide as indicated by the subscript zero.

$$2\mathrm{ClpA} \overset{L_{2,0}}{\rightleftharpoons} \mathrm{ClpA}_2 \tag{23}$$

$$4\mathrm{ClpA} \overset{L_{4,0}}{\rightleftharpoons} \mathrm{ClpA}_4 \tag{24}$$

In our studies, we observed dimers to be significantly populated at only ∼4 and ∼37 °C. Thus, at 25 °C, we predicted $L_{2,0} \leq 1 \times 10^4$ M^{-1} and determined $L_{4,0} = 2.4 \times 10^{14}$ M^{-3}. However, under no conditions did we observe hexamers of ClpA in the absence of nucleotide.

To account for hexamer formation, we can develop a scheme in which dimers and tetramers come together to form hexamers or some other potential pathway. However, because the population of oligomers at thermodynamic equilibrium is independent of path, we can describe the population of hexamers with the equilibrium given in eq 8. In other words, because we do not observe hexamers to be significantly populated at thermodynamic equilibrium, we can model the system by approximating the maximal value of $L_{6,0}$ relative to $L_{2,0}$ and $L_{4,0}$ that would predict a concentration of hexamers that would be below our detection limit. With this in mind, we approximate $L_{6,0}$ to be no larger than 1×10^{24} M^{-5}. With these values of $L_{6,0}$, $L_{4,0}$, and $L_{2,0}$, simulations of species fractions predict that one would not observe hexamers to be significantly populated in our previous sedimentation velocity and

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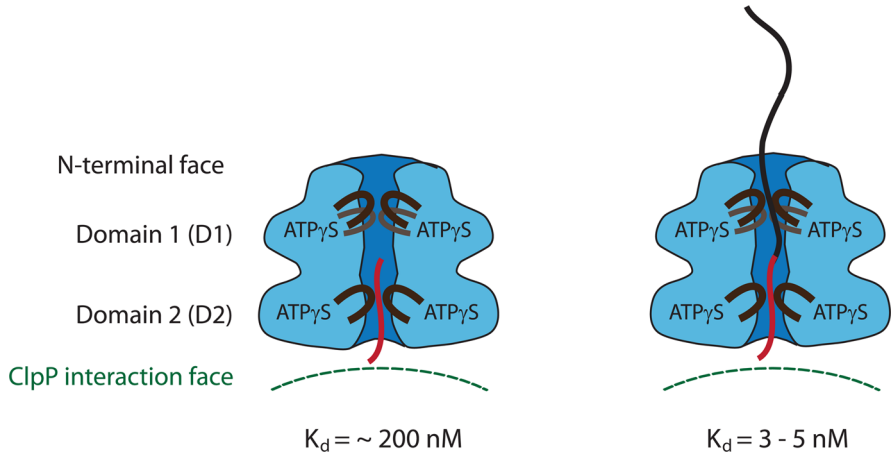

Figure 6. Schematic representation of the domain architecture and interactions with SsrA-containing substrates. ClpA contains three domains from top to bottom they are the N-terminal domain, AAA domain 1 (D1), and AAA domain 2 (D2). On the basis of the work of Hinnerwich et al.,[50] two loops in the axial channel are contributed from D1 and one loop is contributed from D2. The D2 loop interacts with the 11-amino acid SsrA sequence and exhibits a K_d of ~200 nM, whereas when the SsrA sequence is extended by 19 or 39 amino acids, additional contacts in D1 increase the affinity to 3−5 nM.

sedimentation equilibrium experiments in the absence of nucleoside triphosphate (simulations not shown).

In the global analysis of the data presented here, we have included the parameter $L_{6,app}$, which is defined by eq 7. Equation 7 is a function of the hexamerization equilibrium constant in the absence of nucleotide, $L_{6,0}$, scaled by the ratio of partition functions for the binding of nucleotide to hexamers (P_6) to monomers (P_1^6). We have approximated $L_{6,0} \sim 1 \times 10^{24}$ M^{-5} and for binding of ClpA hexamers to N-Cys-50-SsrA $L_{6,app} = (1.4 \pm 0.6) \times 10^{33}$ M^{-5}, indicating that the ratio of partition functions increases the apparent hexamerization constant by a factor of $\sim 1 \times 10^9$ relative to $L_{6,0}$.

From sedimentation velocity experiments performed with ClpA in the presence of 1 mM ATPγS and the absence of a polypeptide substrate, we have determined $L_{6,app} = (5 \pm 1) \times 10^{34}$ M^{-5} (J. Lin, manuscript in preparation), which is similar to the $L_{6,app}$ determined in the polypeptide binding experiments reported here. This observation suggests that the parameter is constrained well in our data analysis. Moreover, because the sedimentation velocity experiments were performed in the absence of peptide, this observation suggests that peptide binding may impact the nucleotide ligation state. In other words, if peptide binding affects the nucleotide ligation state, then the ratio of P_6 to P_1^6 would be different in the presence of peptide and in the absence, which would result in a change in $L_{6,app}$ because $L_{6,0}$ is a constant. On the other hand, the $L_{6,app}$ determined for binding of ClpA to the longer αS1casein substrates is $\sim 10^{39}$ M^{-5}, which represents a 10^6-fold increase compared that of the SsrA substrates. This result suggests that the type of polypeptide substrate bound by ClpA may impact the nucleotide ligation state. Thus, a more direct determination of $L_{6,app}$ as a function of polypeptide substrate is warranted because this parameter may play a role in the apparent specificity for substrates.

The previously reported smaller K_m for α-casein degradation compared to that with SsrA-tagged substrates may be caused by an increase in the population of hexamers. This is because $L_{6,app}$ is larger in the presence of an αS1casein substrate than in the presence of an SsrA-tagged substrate. However, similar impacts on $L_{6,app}$ may be observed in the presence of ClpP. Consequently, an accurate interpretation of kinetic data requires an accounting of all the coupled equilibria in the system. Equally importantly, such a complex array of coupled equilibria likely exists to regulate the activities of ClpA and ClpAP *in vivo*.

Substantial progress in our understanding of the function of a related AAA+ motor, ClpX, has been made through the creation of a linked hexamer.[46] By linking the hexamers into a single continuous protein, Baker, Sauer, and co-workers have eliminated the difficulties of examining such a complex system *in vitro*. However, modulation of substrate recognition and binding through linked equilibria to the assembly state, nucleotide ligation state, and ClpP association may represent an important regulatory component of these enzymes *in vivo*.

Binding to the SsrA Sequence. To the best of our knowledge, the only measurement of binding equilibrium constants comes from ITC and fluorescence experiments performed for binding of ClpA to the 11-amino acid SsrA sequence.[13] In that work, Piszczek and co-workers reported an affinity of 167 ± 83 nM at both 20 and 28 °C. Their data were examined using a 1:1 binding model that did not account for the fact that ClpA resides in a dynamic equilibrium of monomers and hexamers. Surprisingly, when we analyze our data accounting for the assembly state of ClpA, we measure a dissociation equilibrium constant of 200 ± 30 nM, within error of what Piszczek and co-workers reported.

The similarity in the binding equilibrium constant for binding to SsrA measured by us versus that reported by Piszczek and co-workers is likely fortuitous because the experimental conditions are different in the two studies. The measurement of 167 ± 83 nM for binding of ClpA to SsrA by Piszczek and co-workers was taken in 50 mM Tris (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 10% glycerol, and 1−2 mM ATPγS, whereas our measurement of 200 ± 30 nM was taken in 25 mM HEPES (pH 7.5), 300 mM NaCl, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 10% glycerol, and 1 mM ATPγS.

Consistent with our measured binding constant for SsrA being fortuitously the same with the previously reported value by Piszczek and co-workers is the fact that they also reported a binding affinity of 100−312 nM for an SsrA substrate containing an additional 16-amino acid sequence forming an α-helix, with a total length of 27 amino acids. In contrast, the

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SsrA-containing 30- and 50-amino acid substrates examined here exhibited binding constants of ∼3−5 nM, when we account for the assembly state of ClpA and simultaneously analyze four titration isotherms collected at four different polypeptide concentrations. Thus, in this study, we observed that ClpA binds the 11-amino acid SsrA sequence incorporated at the carboxy terminus of a substrate of 30−50 amino acids ∼20-fold stronger than it binds the SsrA sequence alone.

The observation that ClpA binds ∼20-fold tighter to SsrA when it is incorporated into a 30−50-amino acid substrate as opposed to the 11-amino acid SsrA alone suggests that additional nonspecific contacts beyond SsrA are required for optimal binding, a conclusion that was also drawn by Piszczek and co-workers.[13] This observation can be rationalized in the context of the ClpA structure. ClpA is composed of three domains; from the amino terminus to the carboxy terminus, these are an N-domain, AAA D1, and AAA D2.[47] In the hexameric ring, these three domains are stacked with the N-domain at the face where the substrate enters the axial channel, followed by D1 and then D2 arranged in a head-to-tail fashion (see Figure 6).[48] ClpP makes specific contacts with D2. Thus, it is expected that the polypeptide substrate enters from the N-terminal face. Both D1 and D2 contribute loops to the axial channel, termed the D1 and D2 loops. These loops have been hypothesized to be modulated by ATP binding and hydrolysis between up and down states that drive polypeptide translocation.[15,48−50]

Hinnerwisch et al. showed through cross-linking experiments that the D2 loop cross-links to the SsrA sequence at the carboxy terminus of an SsrA-tagged GFP protein.[50] Further, although cross-linking of the substrate to the D1 loops was not observed, mutations in two D1 loops abolished ClpAP-catalyzed translocation and degradation. Moreover, Kress and co-workers showed that ClpA variants that could not hydrolyze ATP at D2 could still translocate the SsrA-tagged substrate using only D1, suggesting D1 maintains a role in polypeptide translocation.[51] Similarly, our examination of the ATP dependence of the rate of polypeptide translocation indicated that two ATP binding and hydrolysis sites are involved in repeating cycles of translocation.[14]

Taken together, these results indicate that during binding the SsrA substrate enters the axial channel from the N-terminal face, passes the D1 loops, and makes direct contact with D2 (see Figure 6). As pointed out by Hinnerwisch and co-workers, in an extended conformation, the 11-amino acid SsrA tag would span ∼35 Å.[50] Further, in their substrate, an additional 8−10 amino acids at the C-terminus are unstructured, spanning an additional 25 Å in the cross channel. They concluded that the 11-amino acid SsrA tag with the additional unstructured 8−10 amino acids was sufficient to span the distance from the interaction site to D1 and D2. With the structural data in mind, the ∼200 nM affinity of binding of ClpA to SsrA likely represents the 11-amino acid SsrA sequence interacting with the D2 loop. The ∼20-fold increase in affinity when the 11-amino acid SsrA sequence is extended to a total of 30 or 50 amino acids is likely the consequence of additional contacts somewhere with the D1 loops.

Here we showed that ClpA in the presence of 1 mM ATPγS was unable to bind to Titin I27 containing SsrA at the carboxy terminus. Similarly, Reid et al. reported that a prebound complex could not be formed with T4 lysozyme containing SsrA at the carboxy terminus in the presence of 1 mM ATPγS but could be formed with λ repressor containing SsrA at the carboxy terminus.[6] Their proposed explanation was that T4 lysozyme is stably folded, whereas the λ repressor is in equilibrium between folded and unfolded states with a time constant of 75 ms. Thus, the most likely explanation for these two observations is that both Titin I27 and T4 lysozyme with SsrA at the carboxy terminus do not provide the additional unstructured region beyond the SsrA sequence to allow enough of the sequence to span the distance from the cross channel in ClpA to the D2 loop. Moreover, in the state of ClpA with ATPγS bound, the energy required to unfold enough of this substrate to provide the additional length is not available.

Binding of ClpA to αS1casein. Wickner and co-workers showed that ClpA would bind to unstructured or poorly structured proteins that do not contain any specific tag sequence.[9] Moreover, Hinnerwisch and co-workers suggested that, perhaps, ClpA binds to apolar regions in proteins and this could be the reason that ClpA binds to SsrA. Here we have shown that ClpA does exhibit modest specificity for the SsrA tag incorporated in a poorly structured polypeptide chain. However, because ClpA binds unstructured proteins, this raises the question of whether multiple ClpA hexamers could bind to polypeptide chains long enough to accommodate more than one hexamer.

To test the possibility that multiple hexamers could bind long peptide chains, we constructed a series of truncations of αS1casein. Full-length αS1casein consists of 214 amino acids and exists in nature as a broad distribution of lengths.[18] Here we show that ClpA binds as a single hexamer to a truncation of αS1casein of 127 amino acids. However, truncations of 152 and 177 amino acids exhibit two hexamers bound. Thus, we conclude that one hexamer binds substrates with ≤127 amino acids and multiple hexamers can bind unstructured substrates with ≥152 amino acids.

It is tempting and desirable to apply the McGhee−von Hippel model for describing binding to a one-dimensional homogeneous lattice.[52] However, we do not currently have a sufficiently long unstructured substrate to determine if a third hexamer can be bound to the substrate. Further, we have only two substrates with which we observe two hexamers bound. On the other hand, observing two hexamers bound raises the question of whether ClpA simply recognizes carboxy and amino termini or if it can recognize internal sequences. Others have shown that ClpAP will degrade substrates containing tag sequences at internal positions that are normally displayed at the ends of the substrate.[53] These observations raise questions about whether ClpA assembles around a binding site or if the substrate enters the axial channel from either the carboxy or amino terminus, which is a topic of future study.

Coupled Equilibria. The fully assembled ATP-dependent protease, ClpAP, is a complex allosteric machine with many levels of coupled equilibria that have not been fully explored. Clearly, the formation of hexamers is coupled to the nucleotide ligation state.[54] Nucleotide-linked assembly is a common feature among many AAA+ protein unfoldases.

We previously showed that ClpA resides in a monomer−dimer−tetramer equilibrium in the absence of nucleotide.[17,26] We further showed that ClpA would assemble into a hexamer with polypeptide binding activity in the presence of ATPγS and, to a lesser extent, AMP-PNP. Also, although hexamers were still formed in the presence of ADP, AMP-PCP, and ADP·BeF, these hexamers were incompetent for polypeptide binding.[22] Thus, it is clear that polypeptide binding is coupled to nucleotide binding. However, the extent to which polypeptide

binding affects the nucleotide ligation state and the ClpA assembly state remains to be seen. In addition, it is also unknown if association of ClpP with ClpA affects both the peptide and nucleotide ligation states. The development of the methods presented here will allow us to begin to quantitatively address these coupled equilibria.

■ ASSOCIATED CONTENT

Supporting Information

Global NLLS analysis of steady-state anisotropy titration data (Figures 1−3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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