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Examination of polypeptide substrate specificity for *Escherichia coli* ClpB

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ABSTRACT

***Escherichia coli* ClpB is a molecular chaperone that belongs to the Clp/Hsp100 family of AAA+ proteins. ClpB is able to form a hexameric ring structure to catalyze protein disaggregation with the assistance of the DnaK chaperone system. Our knowledge of the mechanism of how ClpB recognizes its substrates is still limited. In this study, we have quantitatively investigated ClpB binding to a number of unstructured polypeptides using steady-state anisotropy titrations. To precisely determine the binding affinity for the interaction between ClpB hexamers and polypeptide substrates the titration data were subjected to global non-linear least squares analysis incorporating the dynamic equilibrium of ClpB assembly. Our results show that ClpB hexamers bind tightly to unstructured polypeptides with binding affinities in the range of ~3–16 nM. ClpB exhibits a modest preference of binding to Peptide B1 with a binding affinity of (1.7 ± 0.2) nM. Interestingly, we found that ClpB binds to an unstructured polypeptide substrate of 40 and 50 amino acids containing the SsrA sequence at the C-terminus with an affinity of (12 ± 3) nM and (4 ± 2) nM, respectively. Whereas, ClpB binds the 11-amino acid SsrA sequence with an affinity of (140 ± 20) nM, which is significantly weaker than other polypeptide substrates that we tested here. We hypothesize that ClpB, like ClpA, requires substrates with a minimum length for optimal binding. Finally, we present evidence showing that multiple ClpB hexamers are involved in binding to polypeptides ≥152 amino acids.**

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Key words: chaperones; thermodynamics; binding; protein disaggregation; AAA+ motor proteins; fluorescence spectroscopy; anisotropy; protein unfoldases; translocases; protein quality control.

INTRODUCTION

Molecular chaperones are required to maintain homeostasis in all living cells. The fundamental role of molecular chaperones is to aid other proteins in achieving their final and functional conformations.[1] *Escherichia coli* ClpB is a molecular chaperone that rescues stress-damaged proteins from the aggregated state in collaboration with the DnaK chaperone system (DnaK, DnaJ, and GrpE).[2,3] The survival rate of cells is increased tremendously in the presence of ClpB when cells are under extreme stress, for example, heat shock.[4–6]

ClpB belongs to the Clp/Hsp100 family, which is also known as the Clp ATPases of the AAA+ superfamily (ATPases Associated with various cellular Activities). There are two subclasses in this family defined by the number of conserved AAA+ domains.[7] Proteins in Class 1 have two AAA+ domains, examples include ClpA, ClpB, and ClpC. Whereas Class 2 proteins contain only one AAA+ domain, including ClpX and HslU. Many Clp/Hsp100 chaperones are able to associate with a proteolytic component, for example, ClpP and HslV, to form an ATP-dependent protease, which can degrade unwanted or damaged proteins.[8–10] In contrast, ClpB does not associate with any known peptidase.

A shared feature among Clp/Hsp100 chaperones is that they assemble into oligomeric rings, primarily hexameric rings, in the presence of ATP or ATP analogues.[10–14] ClpB has been found to be monomeric at low protein concentrations in the absence of nucleotides and forms hexamers at high protein concentrations, both in the presence or absence of ATP or ATPγS.[15] Both previous published data [15,16] and our data (J. Lin, manuscript in preparation) suggest that ClpB hexamers reside in a dynamic equilibrium with monomers. Thus, it is important to consider the

Additional Supporting Information may be found in the online version of this article.

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dynamic equilibrium of ClpB self-assembly in quantitative measurements of ClpB activities.

In previous studies on ClpB binding to polypeptide substrate, it has been assumed that the total concentration of ClpB monomers resides in the hexameric state.[17,18] Consequently, the concentration of hexamers present and available to bind to a polypeptide substrate may not be correct. This uncertainty in the concentration will lead to uncertainties in the measured binding equilibrium constants and thus our ability to predict the concentration of bound complex.

The structure of ClpB is similar to that of ClpA. Both ClpA and ClpB are composed of an N-domain followed by two AAA+ domains. The primary difference between ClpA and ClpB is that the two AAA+ domains in ClpB are separated by an alpha helical region termed the M-domain. The two tandem AAA+ domains conserved in ClpA and ClpB are termed Domain 1 (D1) and Domain 2 (D2). Both D1 and D2 are Walker-type nucleotide binding domains with conserved Walker A and Walker B motifs, which are responsible for binding and hydrolysis of nucleotides. A conserved region located between each Walker A and Walker B motif is referred to as the pore loop. When ClpB or ClpA forms the ring-shaped hexamer, the D1 pore loop and D2 pore loop are facing into the axial channel of the hexamer.[18,19] It has been shown that both D1 and D2 pore loops are critical for polypeptide substrate interaction in both ClpB and ClpA.[13,17,18,20–22]

Many Clp/Hsp100 chaperones are able to recognize specific sequences located at either the N- or C-terminus of polypeptide substrates.[9,23] For example, ClpA and ClpX both recognize the SsrA sequence, which is an 11 amino acid sequence that is cotranslationally incorporated into the C-terminus of partially synthesized polypeptides on stalled ribosomes.[24] Similarly, RepA, the P1 plasmid initiator protein, can be specifically recognized by ClpA through the first 15 amino acids at its N-terminus.[25]

Our understanding of ClpB substrate recognition is limited. Previous published studies show that ClpB can bind to unstructured polypeptides (such as caseins) and thermal or chemical induced protein aggregates.[2,17] In an attempt to further identify ClpB binding sequences, Schlieker and colleagues screened 1,106 cellulose-bound peptides representing sequences from six full length proteins known to interact with ClpB.[17] From that work they identified a 21-amino acid sequence, Peptide B1, which is bound by ClpB and is enriched with positively charged and aromatic residues.

To our knowledge, few quantitative studies on ClpB substrate recognition have been reported. Schlieker *et al.* made a qualitative estimate of the upper limit of the binding affinity for ClpB binding to Peptide B1 of less than 80 nM in the presence of ATPγS based on the observation that the increase in peptide fluorescence plateaued at a 1:1 ratio of polypeptide and ClpB hexamers both at a concentration of 80 nM. Again, assuming all of the ClpB was hexameric.[17] Although estimating an upper limit on the binding constant in this way may be warranted, a more quantitative examination of the binding is needed in order to fully understand substrate specificity. In that study, they also constructed a ClpB variant with mutations in the Walker B motif of both AAA+ domains, termed ClpB-B1/2A, which can bind but not hydrolyze ATP. They reported a binding affinity of $K_d = (23 \pm 2)$ nM for ClpB-B1/2A binding to Peptide B1 in the presence of hydrolysable ATP. However, all the values were obtained under the assumption that ClpB is not in a dynamic equilibrium of monomers and hexamers. Rather, all of the ClpB was assumed to reside in the hexameric state. Moreover, although it was shown that the ClpB variant forms hexamers using size exclusion chromatography, the extent to which the mutation perturbs the monomer to hexamer equilibrium was not addressed.

Here, we present the first quantitative study of ClpB-substrate binding. In our study, the dynamic equilibrium of ClpB self-assembly is incorporated into the examination of ClpB binding to its target substrates. For this study, we used the same set of polypeptide substrates that we previously used to examine the ClpA-polypeptide binding activity. In that study we reported CD spectra that indicated these substrates are unstructured.[26] Here, we report that ClpB hexamers exhibit a $K_{d,6}$ in the range of ~3–16 nM for most substrates. On the other hand, the binding affinity for Peptide B1 was determined to be $K_{d,6} = (1.7 \pm 0.2)$ nM, which represents a modest increase in the specificity relative to other polypeptides examined here. Thus, our results indicate that ClpB exhibits little difference in binding affinity to a variety of unstructured polypeptides, regardless of their sequences. We also observed that multiple ClpB hexamers bind to unstructured polypeptides equal to or longer than 152 amino acids. Taken together, we hypothesize that ClpB may differentiate protein aggregates from native proteins based on the length of exposed unstructured regions.

MATERIALS AND METHODS

Reagents and buffers

All chemicals were reagent grade. All buffers were prepared with distilled and deionized water produced from Purelab Ultra Genetic system (Siemens Water Technology, Germany). Buffer H200 is 25 mM HEPES pH 7.5 at 25°C, 200 mM NaCl, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 10% (v/v) glycerol, where the 200 indicates the NaCl concentration.

Plasmid, protein and peptides

E. coli ClpB was purified as described.[27] The recombinant plasmids for αS1casein truncations were

constructed as described.[28] Purification of the αS1casein truncations were performed as described.[28] Peptide B1 was synthesized by AnaSpec (Fremont, CA). All other polypeptides with lengths ranging from 11 amino acids to 50 amino acids were synthesized by CPC Scientific (Sunnyvale, CA). All peptides were certified >96% pure based on reverse phase HPLC and the mass was confirmed by mass spectrometry. All polypeptides were labeled with Fluorescein-5′-maleimide from Life Technologies (Carlsbad, CA) as described.[28]

Analytical ultracentrifugation

Analytical ultracentrifugation experiments were performed using a Beckman Optima XL-A analytical ultracentrifuge. Sedimentation velocity experiments were performed by loading a 380 μL sample of protein into a double sector Epon charcoal-filled centerpiece and the sample was subjected to an angular velocity of 40,000 rpm. Absorbance scans as a function of radial position were collected by scanning the sample cells at a wavelength of either 290 nm or 494 nm in continuous scanning mode. c(s) distributions were determined by analyzing the raw data using Sedfit (Peter Schuck, NIH). For direct boundary fitting the data were analyzed with SedAnal.

Anisotropy titration experiments

Steady-state anisotropy titrations were performed by titrating fluorescently modified polypeptide with ClpB in buffer H200 at 25°C. Both the titrant and the sample in the cuvette were in the presence of 1 mM ATPγS to insure that the concentration of nucleotide remained constant throughout the titration. The incubation time for each titration point to achieve equilibrium was determined by fluorescence stopped-flow experiments for each substrate, which was found to be ~20–30 min. Anisotropy signal changes were monitored by exciting fluorescein at $\lambda_{ex} = 494$ nm and observing emission at $\lambda_{em} = 515$ nm with a Fluorolog-3 spectrofluorometer (HORIBA Jobin Yvon, Japan). ClpB has been reported to hydrolyze ATPγS at a rate of ~0.2 min^{-1} $hexamer^{-1}$.[29] Assuming all of the ClpB is hexameric and thirty minutes between titration points we predict that ~0.020 mM ATPγS is hydrolyzed out of 1 mM ATPγS by the end of the titration.

Competition steady-state anisotropy titrations were performed by titrating 110 nM Flu-N-Cys-50-SsrA in the presence of varying concentrations of polypeptide substrate lacking a fluorophore with a solution of ClpB also containing 110 nM fluorescently modified polypeptide substrate. The fluorescently modified substrate and 1 mM ATPγS were included in both the cuvette and the titrant to insure that a fixed concentration of both components was maintained throughout the titration.

Fluorescence anisotropy measurements were performed with the L-format excitation and emission arrangement as we have previously reported.[26] For a detailed presentation see Lakowicz[30] for further review of the method see Licata *et al.*[31] Briefly, the anisotropy, *r*, is calculated according to Eq. 1,

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

where "*I*" represents the emission intensity and the first and second subscript represent the orientation of the excitation and emission polarizer, respectively. *G* is the G-factor, which is calculated as $G = I_{HV}/I_{HH}$, where the notation is the same as for Eq. 1.

Steady-state anisotropy titration data are plotted as relative anisotropy increase, $\Delta r/r_0$ versus total ClpB monomer concentration, $[ClpB]_T$. Relative anisotropy increase is determined based on Eq. 2

$$\frac{\Delta r}{r_0} = \frac{r_{obs} - r_0}{r_0} = \frac{r_{obs} - r_f}{r_f} \quad (2)$$

where r_{obs} is the observed anisotropy, r_0 is the initial anisotropy, and r_0 is equal to the anisotropy of free fluorescently modified peptide, r_f.

Binding density function analysis

Each set of titrations collected at four different total polypeptide concentrations was subjected to the Binding Density Function (BDF) analysis as previously described.[32–35] This was done to determine the dependence of the relative anisotropy increase on the extent of binding $\overline{X}(([ClpB]_{monomer\ bound})/[Peptide]_T)$ and the maximum stoichiometry at saturating [*ClpB*]. The 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. Since 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 ($[ClpB]_T$) was required to achieve the observed signal change. If the thermodynamic state of the macromolecule is the same then the extent of binding, $\overline{X}$, must be the same.[32–35] Since the extent of binding is governed by the free ligand concentration ($[ClpB]_f$) the free ClpB concentration at each point of intersection must also be the same. Thus, to determine the extent of binding, the total ClpB 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. 3,

$$[ClpB]_T = \overline{X}[Peptide]_T + B \quad (3)$$

where $[Peptide]_T$ represents the independent variable, $[ClpB]_T$ represents the dependent variable, the extent of

binding, $\overline{X}$, represents the slope, and B represents the y-intercept, which would be the sum of all of the terms containing $[\mathrm{ClpB}]_f$ given by Eq. 4.

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

From this analysis, a value of the extent of binding $\overline{X}$ 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 $\overline{X}$ is constructed to determine the relationship between the signal change and the extent of binding and predict the maximum stoichiometry at saturating ClpB concentrations.-That is to say, the maximum stoichiometry is determined by extrapolating the plot of signal vs. extent of binding to the maximum observed Relative Anisotropy Increase and determining the value of the extent of binding on the x-axis at this point.

In many cases the relationship between the relative anisotropy increase and the extent of binding, $\overline{X}$, was found to be nonlinear. Consequently, the relationship between signal change and extent of binding was analyzed using the empirical function approach as previously described.[34,36,37] In this approach, the relationship between the relative anisotropy increase, $\Delta r/r_0$, and the extent of binding, $\overline{X}$, is described by an empirical function, typically a polynomial, based on the shape of the relative anisotropy increase vs. extent of binding. In this study, the relationship was well described by a second degree polynomial given by Eq. 5

$$\frac{\Delta r}{r_0}=a+b\overline{X}+c\overline{X}^2 \quad (5)$$

Where a, b, and c represent fitting parameters.

NLLS analysis

The binding association equilibrium constant for ClpB hexamers binding to peptides, K_6, is defined by the equilibrium in Eq. 6 and $K_{\mathrm{d},6}=1/K_6$.

$$\mathrm{ClpB_6}+\mathrm{Peptide}\overset{K_6}{\rightleftharpoons}\mathrm{ClpB_6\text{-}Peptide} \quad (6)$$

The apparent self-assembly constant for ClpB hexamer, $L_{6,\mathrm{app}}$, is defined by the equilibrium in Eq. 7,

$$6\{ClpB\}\overset{L_{6,\mathrm{app}}}{\rightleftharpoons}\{ClpB_6\} \quad (7)$$

where $\{ClpB\}$ and $\{ClpB_6\}$represent the summation of all of the nucleotide ligation states of monomeric and hexameric ClpB, respectively.

The apparent self-assembly constants for ClpB dimer and tetramer, $L_{2,\mathrm{app}}$ and $L_{4,\mathrm{app}}$, are defined by the equilibrium in Eqs. 8 and 9, respectively,

$$2\{ClpB\}\overset{L_{2,\mathrm{app}}}{\rightleftharpoons}\{ClpB_2\} \quad (8)$$

$$4\{ClpB\}\overset{L_{4,\mathrm{app}}}{\rightleftharpoons}\{ClpB_4\} \quad (9)$$

where $\{ClpB_2\}$ and $\{ClpB_4\}$represent the summation of all of the nucleotide ligation states of dimeric and tetrameric ClpB, respectively.

Competition titrations were subjected to NLLS analysis using Eqs. 5, 10, and 11. Equation 10 is the conservation of mass equation that accounts for binding to the competitor given by the extent of binding to the competitor, $\overline{X_C}$, times the total competitor concentration, $[\mathrm{Competitor}]_T$.

$$[\mathrm{ClpB}]_T=[\mathrm{ClpB}]_f+2L_{2,\mathrm{app}}[\mathrm{ClpB}]_f^2+4L_{4,\mathrm{app}}[\mathrm{ClpB}]_f^4+6L_{6,\mathrm{app}}[\mathrm{ClpB}]_f^6+\overline{X}[\mathrm{Peptide}]_T+\overline{X_C}[\mathrm{Competitor}]_T \quad (10)$$

The extend of binding to competitor, $\overline{X_C}$, is given by Eq. 11.

$$\overline{X_C}=\frac{[\mathrm{ClpB}]_b}{[\mathrm{Competitor}]_T}=\frac{6[\mathrm{ClpB_6\text{-}Competitor}]}{[\mathrm{Competitor}]_T}=\frac{6K_{6C}L_{6,\mathrm{app}}[\mathrm{ClpB}]_f^6}{1+K_{6C}L_{6,\mathrm{app}}[\mathrm{ClpB}]_f^6} \quad (11)$$

$K_{6\mathrm{C}}$ is the binding equilibrium constant which is defined by the equilibrium in Eq. 12 and $K_{\mathrm{d},6\mathrm{C}}=1/K_{6\mathrm{C}}$.

$$\mathrm{ClpB_6}+\mathrm{Competitor}\overset{K_{6C}}{\rightleftharpoons}\mathrm{ClpB_6\text{-}Competitor} \quad (12)$$

RESULTS

Examination of ClpB binding αS1casein

To begin to examine the specificity of ClpB binding to polypeptide substrates we performed quantitative anisotropy titrations as recently described for *E. coli* ClpA (see Materials and Methods).[28] To this end, we examined binding to the intrinsically unstructured protein (IUP) αS1casein, which is a natural substrate for ClpB.[38] In our previous study we reported CD spectra for these substrates that is consistent with a random coil.[26] The full length αS1casein is 214 amino acids. We modified and reconstructed αS1casein to contain a single cysteine at the amino terminus for incorporation of Fluorescein-5'-maleimide.[28] The resulting substrate, αS1casein-177, contains 177 amino acids and one cysteine residue at the

Table I
Polypeptide substrates

Name	Length (AA)	Sequence or Source
αS1casein-177	177	C-terminal 177 AA of αS1-casein with N-terminal cysteine
αS1casein-152	152	C-terminal 152 AA of αS1-casein with N-terminal cysteine
αS1casein-127	127	C-terminal 127 AA of αS1-casein with N-terminal cysteine
αS1casein-102	102	C-terminal 102 AA of αS1-casein with N-terminal cysteine
N-Cys-50-SsrA	50	CLILHNKQLGMTGEVSFQAANTKSAANLKVKELRSKKKLAANDENYALAA
N-Cys-50	50	CEIIEDGKKHILILHNKQLGMYTGEVSFQAANTKSAANLKVKELRSKKKL
N-Cys-40-SsrA	40	CTGEVSFQAANTKSAANLKVKELRSKKKLAANDENYALAA
SsrA	11	AANDENYALAA
Peptide B1	21	AHAWQHQGKTLFISRKTYRIC

amino terminus, see Table I. Upon labeling with Fluorescein the substrate is denoted as Flu-αS1casein-177.

Anisotropy titrations were performed by titrating 57, 114, 171, and 228 nM Flu-αS1casein-177 with ClpB in H200 at 25°C (see Materials and Methods). Similar to what we have observed for ClpA,[26] the titration curves appear steeper than would be expected for a simple 1:1 binding interaction, that is, 1 hexamer binding one polypeptide [see Fig. 1(A)]. This observation may indicate that ClpB resides in a dynamic equilibrium of monomers and hexamers. Alternatively, the steepness of the curves could indicate that multiple monomers or various oligomeric states are binding to the polypeptide substrate.

To determine the number of monomers bound to Flu-αS1casein-177 we subjected the anisotropy titration curves to the binding density function analysis (BDF)[28,35] (see Materials and Methods). The BDF analysis is a model independent method for determining the maximum stoichiometry of binding for a noninteracting macromolecule. In this case, the macromolecule is the Flu-αS1casein-177 substrate and ClpB is the ligand. Even though the ligand, ClpB, may exist in a number of assembly states the analysis still applies.[26,39] The results of the BDF analysis are shown in Figure 1(B), where Figure 1(B) is a plot of the Relative Anisotropy Increase [$\Delta r/r_0$, see Eq. 2] vs. the extent of binding ($[\text{ClpB}]_{\text{monomer bound}}/[\text{Flu-}\alpha\text{S1casein-177}]_T$). The four titration curves shown in Figure 1(A) all saturate at a maximum relative anisotropy increase value of ~0.83, which is represented by a solid horizontal line in Figure 1(B) (see Supporting Information Table SI for absolute anisotropy values). The BDF analysis was found to be reliable over a range of relative anisotropy from ~0.3 to 0.6. Consequently, a short extrapolation to the maximum value of 0.83 indicates a maximum binding stoichiometry of approximately 24 ClpB monomers per polypeptide substrate, Figure 1(B). This result suggests that many more monomers are bound than would be expected for a single hexamer, that is, 6 monomers per hexamer. This result may suggest that multiple ClpB hexamers are bound per 177 amino acid substrate.

Multiple ClpB hexamers bind to long unstructured polypeptide substrates

Sedimentation velocity experiments were performed to test the hypothesis that more than one ClpB hexamer may bind to long unstructured polypeptides. First, 4.3 μM Flu-αS1casein-177 was subjected to centrifugation and absorbance was monitored at 494 nm where the fluorescein dye absorbs. The sedimentation velocity absorbance boundaries were subjected to analysis using Sedfit and a c(s) distribution was generated [see Fig. 2(A)]. The c(s) distribution shows a broad reaction boundary between 1 and 5 S for the 177 amino acid αS1casein truncation.

The broad reaction boundary for the 177 amino acid αS1 casein substrate might suggest that the substrate is aggregated. Such aggregation would violate the assumptions in the BDF analysis since the analysis requires that the macromolecule does not aggregate. Thus, a direct boundary fit was performed on the data to further probe for the presence of aggregates. The direct boundary fit shown in Figure 2(B) reveals that the data are best described by a single component model and there is no indication of aggregation. The analysis yields a mw = (18.4 ± 0.2) kDa, which is in reasonably good agreement with the predicted molecular weight of 20.5 kDa from sequence.

It is important to note that the 4.27 μM Flu-αS1casein-177 used in this sedimentation velocity experiment is ~20 fold higher in concentration than the highest concentration used in the anisotropy titrations (~200 nM). The elevated concentration in the centrifugation experiment is necessary to improve the signal to noise since absorbance is being used to detect the sedimentation. However, the higher concentration would favor aggregation, if present. On the other hand, the observed anisotropy values for 57, 114, 171, and 228 nM Flu-αS1casein-177 were 0.1239, 0.1238, 0.1249, 0.1247, respectively (see Supporting Information Table SI). This indicates that the anisotropy value is independent of protein concentration, which is consistent with no increased aggregation over the four-fold concentration range where the anisotropy titrations were carried out. Finally, the

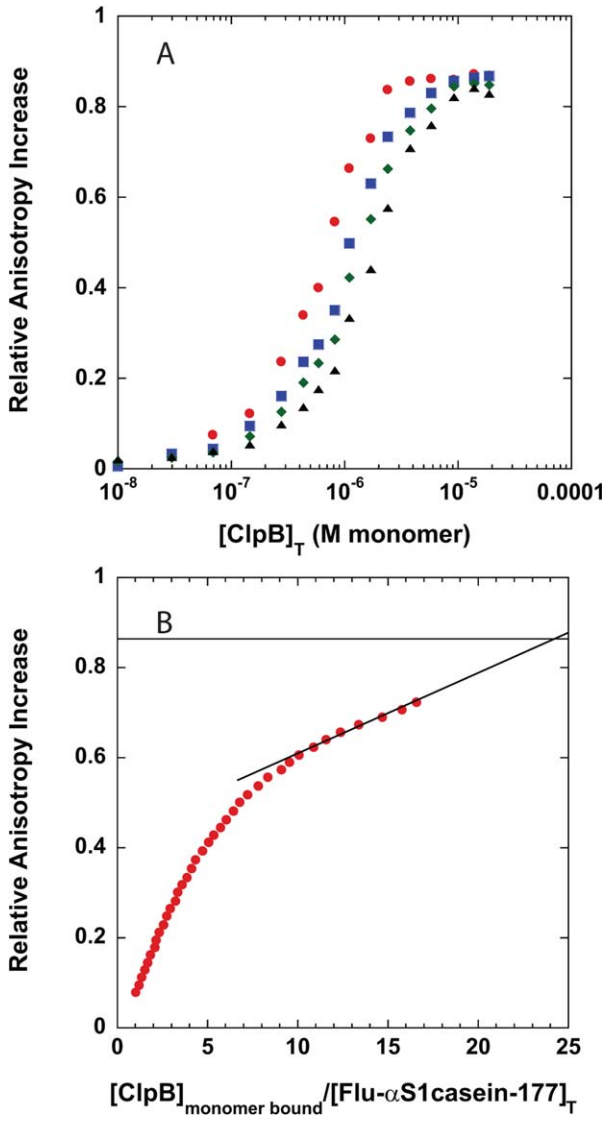

Figure 1

Steady-state anisotropy titrations of Flu-αS1casein-177 with ClpB in the presence of 1 mM ATPγS in buffer H200 at 25°C. (A) Relative Anisotropy Increase for the titrations with 57 nM (●), 114 nM (■), 171 nM (◆), and 228 nM (▲) Flu-αS1casein-177 titrated by ClpB. (B) Relative Anisotropy Increase as a function of the extent of binding ($[ClpB]_{monomer\text{-}bound}/[Flu\text{-}\alpha S1casein\text{-}177]_T$) determined from the binding density function (BDF) analysis of the titrations presented in panel A. The solid horizontal line represents the maximum observed signal increase for the four titrations in panel A of ~0.83. The sloped solid line represents an extrapolation intersects the horizontal line (maximum signal change) at ~ 24 ClpB monomers bound per polypeptide substrate.

protein concentration independent average of these four values is $r = 0.1243 \pm 0.0005$, which represents an ~0.4 % fluctuation, which is consistent with no change in the aggregation state over the protein substrate concentration examined. In fact, for all substrates examined by anisotropy titrations, over the four-fold concentration range (~50 nM–200 nM) the average initial anisotropy changes by less than 0.5 % (see Supporting Information Table SII). Leading us to conclude that there is no evidence for aggregation. Finally, the identical sedimentation velocity experiment was performed with αS1casein-102 and neither the c(s) data nor the direct boundary fit are consistent with aggregation, (see Supporting Information Fig. S1).

Next, 25 μM ClpB monomer in the presence of 1 mM ATPγS was subjected to centrifugation and absorbance was monitored at 290 nm. These data were also analyzed with Sedfit and the c(s) distribution is shown in Figure 2(C). At these relatively high protein and nucleotide concentrations, the c(s) distribution shows that ClpB exists in solution predominantly as a single oligomer with a sedimentation coefficient of ~15.5 S, consistent with the majority of ClpB residing in the hexameric state at a concentration of 25 μM ClpB monomer.

To examine the binding of hexameric ClpB to Flu-αS1casein-177, sedimentation velocity experiments were performed with 25 μM ClpB monomer and 4.27 μM Flu-αS1casein-177 in the presence of 1 mM ATPγS. The absorbance of the fluorescein attached to αS1casein was monitored at 494 nm. Thus, the only components observed are free Flu-αS1casein-177 and ClpB that is bound to Flu-αS1casein-177. However, since ClpB doesn't absorb at 494 nm, ClpB that is not bound to the polypeptide substrate will not contribute to the signal. The absorbance boundaries were subjected to analysis with Sedfit and the c(s) distribution is shown in Figure 2(D).

The c(s) distribution in Figure 2(D) exhibits a reaction boundary at ~1–5 S consistent with unbound Flu-αS1casein-177, compare Figure 2(A) to Figure 2(D). Also, the c(s) distribution exhibits a broad distribution between ~15 S and 30–35 S. Since ClpB in the presence of 1 mM ATPγS in the absence of polypeptide exhibits a tight distribution centered at ~15.5 S [see Fig. 2(C)], the broad distribution observed when monitoring ClpB bound to the substrate indicates that hexamers and, potentially, a variety of other oligomeric states are bound to the substrate. In other words, the broadness of this distribution suggests that a combination of Flu-αS1casein-177 bound by one ClpB hexamer and Flu-αS1casein-177 potentially bound by multiple hexamers. Consequently, the sedimentation velocity experiments are consistent with something larger than a hexamer binding the substrate. However, because of the very broad distribution, little more than something larger than a hexamer can be concluded from these data, which is consistent with the BDF analysis in Figure 1(B).

Minimal substrate length to accommodate one ClpB hexamer

Observing multiple ClpB oligomers bound to the 177 amino acid substrate suggests nonspecific binding to a linear lattice similar to the McGhee-von Hippel model

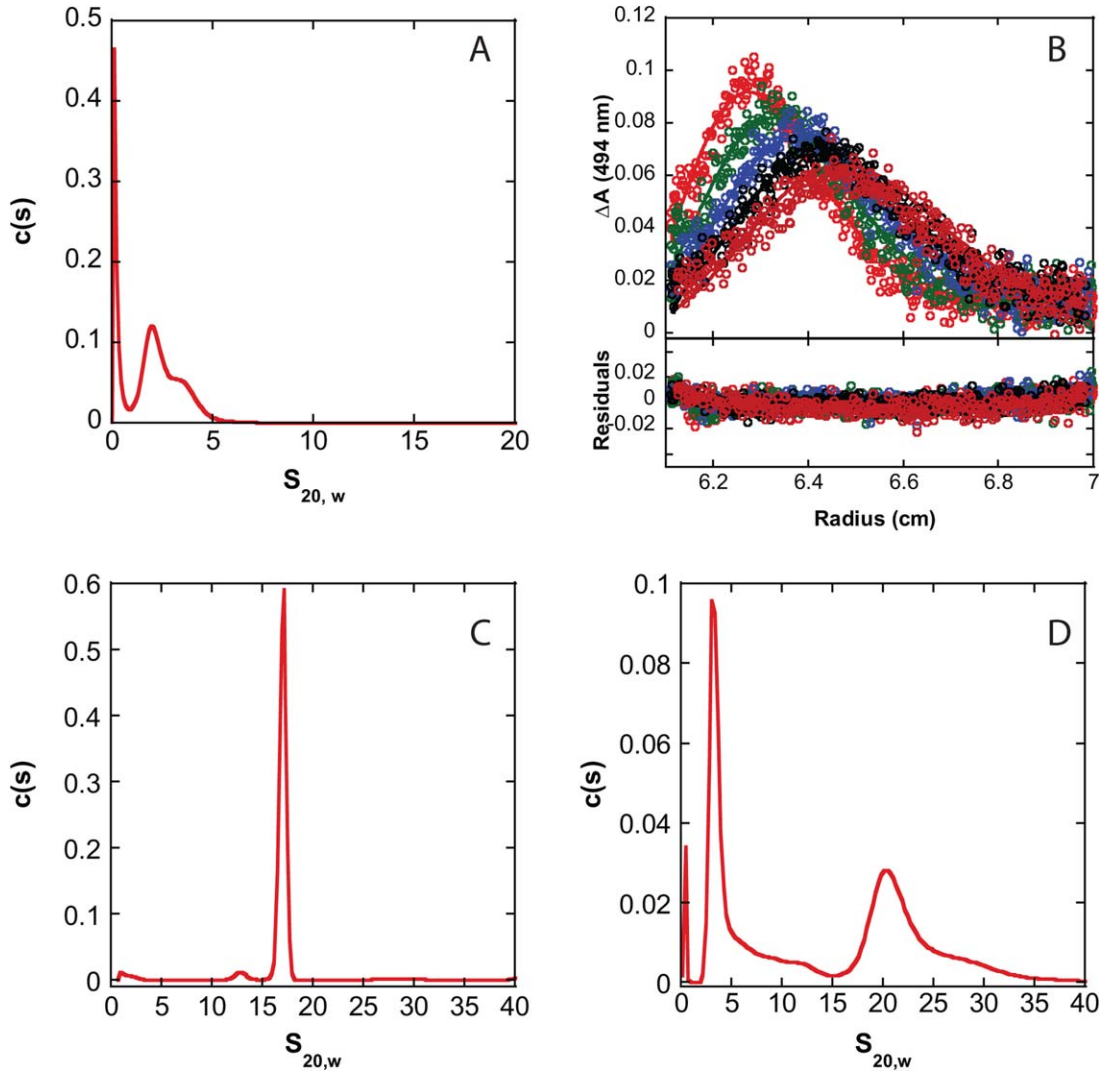

Figure 2

Sedimentation velocity experiments with 4.2 μM Flu-αS1casein-177 in H200 at 25°C monitored at 494 nm analyzed with (A) SedFit to determine the sedimentation coefficient distribution, c(s), or (B) SedAnal to perform direct boundary fitting on the difference curves to a single component model yielding a molecular weight of mw = (18.4 ± 0.2) kDa, (C) 25 μM ClpB in the presence of 1 mM ATPγS in Buffer H200 at 25°C monitored at 290 nm, and (D) 25 μM ClpB and 4.2 μM Flu-αS1casein-177 in the presence of 1 m*M* ATPγS in Buffer H200 at 25°C monitored at 494 nm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

for nonspecific protein-nucleic acid interactions.[40] In this model, the length of substrate required to accommodate a second hexamer would be governed by the occluded site size of the enzyme. However, unlike the application of this model to protein-nucleic acid interactions, the linear lattice that ClpB binds is inhomogeneous in sequence and likely structure. Nevertheless, one basic prediction of this model is that a sufficiently short substrate should only accommodate a single hexamer of ClpB.

To determine the length of the αS1casein truncations that will accommodate a single ClpB hexamer, we constructed a series of C-terminal truncations derived from αS1casein-177 of various lengths resulting in αS1casein-152, αS1casein-127, and αS1casein-102 (see Table I for substrate definitions). All substrates were labeled with fluorescein at the amino terminus, again, denoted as Flu-αS1casein-152, Flu-αS1casein-127, and Flu-αS1casein-102, respectively (Table I). Steady-state anisotropy titration experiments were performed to examine the binding of ClpB to these substrates. As described above, each substrate was titrated with ClpB at four different concentrations of modified αS1casein substrate as indicated in the legend of Figure 3. The titration isotherms are similar to the titration isotherms for Flu-αS1casein-177 shown in Figure 1(A), where the steepness of the curves indicates there is some cooperative linkage between binding of each ClpB monomer or oligomer.

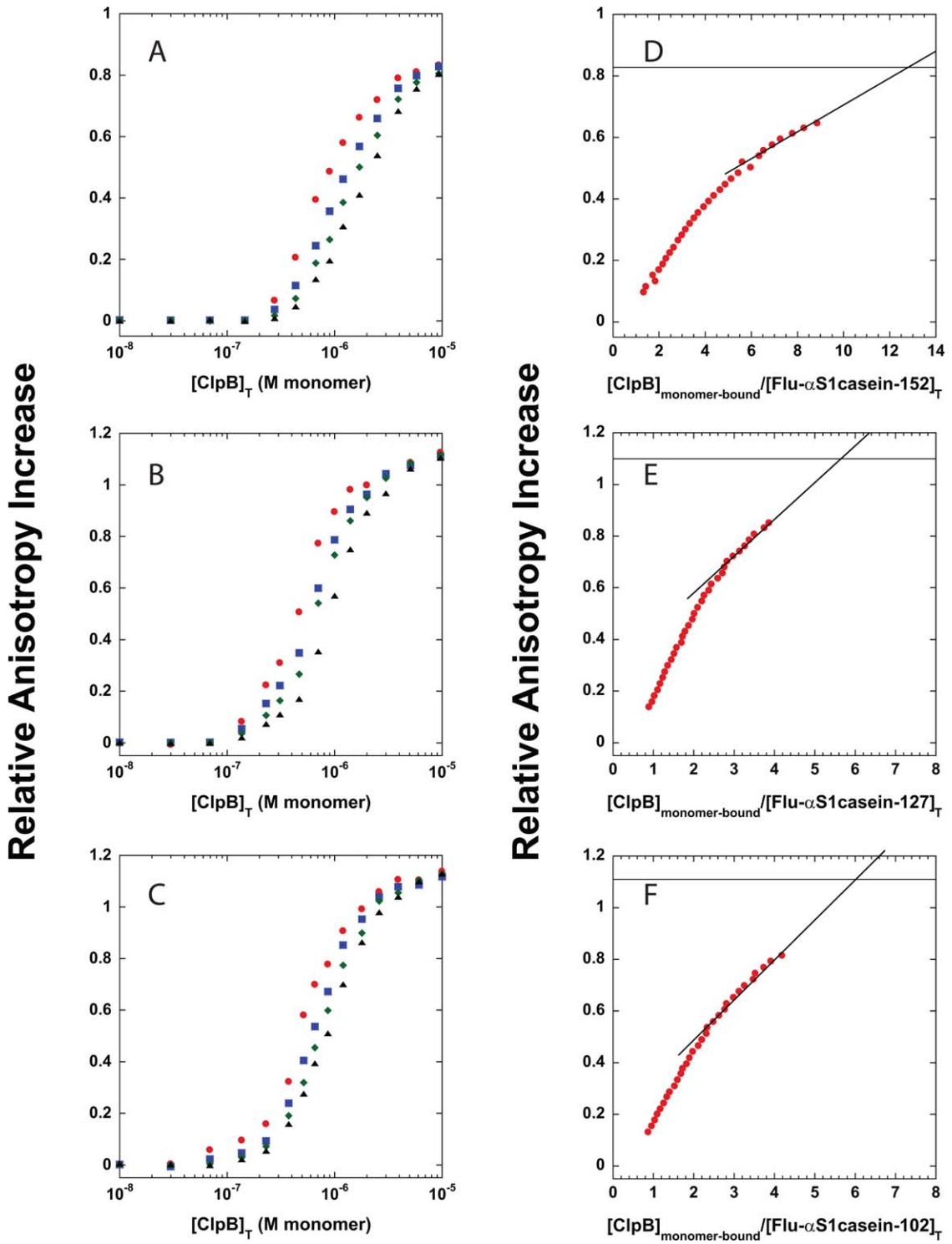

Figure 3

Steady-state anisotropy titration data for (A) Flu-αS1casein-152 collected at 70 nM (●), 140 nM (■), 210 nM (♦), and 280 nM (▲) (B) Flu-αS1casein-127 collected at 73 nM (●), 145 nM (■), 217 nM (♦), and 290 nM (▲) (C) Flu-αS1casein-102 collected at 52 nM (●), 105 nM (■), 157 nM (♦), and 209 nM (▲) titrated with ClpB. Panel (D), (E) and (F) are showing the BDF analysis of steady-state anisotropy data presented in panel (A), (B) and (C), respectively. The black solid horizontal lines in panel (D), (E) and (F) represent the maximum observed signal increase.

Each set of four titrations for each αS1casein length was subjected to BDF analysis and the results are plotted in Figure 3(D–F). For all three substrates the dependence of the relative anisotropy increase on stoichiometry is observed to be nonlinear. To approximate the maximum binding stoichiometry, the second phase of the data were used to extrapolate to the maximum signal change, which is indicated by the horizontal solid line.

For Flu-αS1casein-152, BDF analysis shows that the maximum binding stoichiometry is ∼ 12 monomers or two hexamers [see Fig. 3(D)]. This result indicates that, similar to the 177 amino acid substrate, there are more than six monomers bound to the 152 amino acid substrate. In contrast, anisotropy titrations and subsequent BDF analyses for both Flu-αS1casein-127 and Flu-αS1casein-102 show that the maximum binding stoichiometry is ∼6 ClpB monomers per single polypeptide [Fig. 3(E,F)]. All in all, these results are consistent with the hypothesis that more than one ClpB hexamer is able to bind to αS1casein substrates of 152 amino acids and above, whereas, only one hexamer is observed to bind to substrates shorter than 127 amino acids.

Global NLLS analysis of ClpB-substrate binding isotherms

Since the αS1casein-102 is sufficiently short to bind only one hexamer, we subjected the titration curves shown in Figure 3(C) to global NLLS analysis to determine the best model that describes the data. If all of the ClpB monomers are in the hexameric state under our experimental conditions and six monomers are observed to bind to a given substrate then the set of titration curves collected at four different αS1casein substrate concentrations should be described by a simple 1:1 binding model. In this model, all of the ClpB would be assumed to be in the hexameric state and only hexamers interact with polypeptide. To test this, the anisotropy titrations performed with Flu-αS1casein-102 [Fig. 3(C)], were subjected to global NLLS analysis using Eqs. 5, 13, and 14

$$\overline{X} = \frac{K_6 x_6}{1 + K_6 x_6} = \frac{K_6 [\mathrm{ClpB_6}]_f}{1 + K_6 [\mathrm{ClpB_6}]_f} \tag{13}$$

where K_6 represents the binding equilibrium constant for binding ClpB hexamers to the polypeptide [see Eq. 6 in Materials and Methods], and $[ClpB_6]_f$ represents the concentration of free ClpB hexamers, which is determined based on the conservation of mass equation given by Eq. 14.

$$\frac{[\mathrm{ClpB}]_T}{6} = [\mathrm{ClpB_6}]_T = \overline{X}[\mathrm{Peptide}]_T + [\mathrm{ClpB_6}]_f \tag{14}$$

In Eq. 14, $[\mathrm{ClpB}]_T$ represents the known total ClpB monomer concentration, $[\mathrm{ClpB_6}]_T$ is the total hexamer concentration based on dividing the total monomer concentration by 6, which assumes only hexamers reside in solution, and $[Peptide]_T$ represents the known total polypeptide concentration. This model can be considered as a basic 1:1 macromolecule (peptide) to ligand (hexameric ClpB) binding model. The fitting results are shown in Figure 4(A), and this model clearly does not adequately describe all four titration isotherms. This observation is inconsistent with all of the ClpB residing in the hexameric state.

Since the model that assumes that all of the ClpB monomers are in the hexameric state cannot describe the data, the data were subjected to NLLS analysis using the n-independent and identical sites model.[41] In this model, all step-wise microscopic binding constants, k, for binding of each ClpB monomer are equal. Thus, the extent of binding, $\overline{X}$, can be calculated as shown in Eq. 15.

$$\overline{X} = \frac{nkx}{1 + kx} = \frac{6k[\mathrm{ClpB}]_f}{1 + k[\mathrm{ClpB}]_f} \tag{15}$$

where x represents the ligand, which, in this case, is the free monomer of ClpB given by $[ClpB]_f$. Since the BDF analysis presented in Figure 3(F) shows that six monomers are bound to the substrate (Flu-αS1casein-102) at equilibrium, n in Eq. 15 was constrained to 6. The conservation of mass equation is given by Eq. 16,

$$X_T = [\mathrm{ClpB}]_T = \overline{X}[\mathrm{Peptide}]_T + [\mathrm{ClpB}]_f \tag{16}$$

where, $[\mathrm{ClpB}]_T$ represents the total monomer concentration of ClpB. The data in Figure 3(C) were subjected to global NLLS analysis with Eqs. (5, 15, and 16). The smooth solid lines shown in Figure 4(B) represent the best fit and are not sufficiently steep to describe the data. The steepness of the data indicates that there could be positive cooperativity between binding of each ClpB monomer.

Since both the simple 1:1 binding model and the n-independent and identical sites binding model cannot describe our data, and the titration data exhibit a shape that is consistent with positive cooperativity, we next tested the infinite cooperative binding model. In this model, the only species are unbound peptide, free ClpB monomer and peptide bound with six ClpB monomers. The infinite cooperative model assumes that there are no intermediate bound states, for example, two monomers bound state, three monomers bound state, etc. In this model, the extent of binding, $\overline{X}$, is expressed in Eq. 17.

$$\overline{X} = \frac{n(kx)^n}{1 + (kx)^n} = \frac{6(k[\mathrm{ClpB}]_f)^6}{1 + (k[\mathrm{ClpB}]_f)^6} \tag{17}$$

where, n is the Hill coefficient and was constrained to 6. The same set of data in Figure 3(C) were subjected to

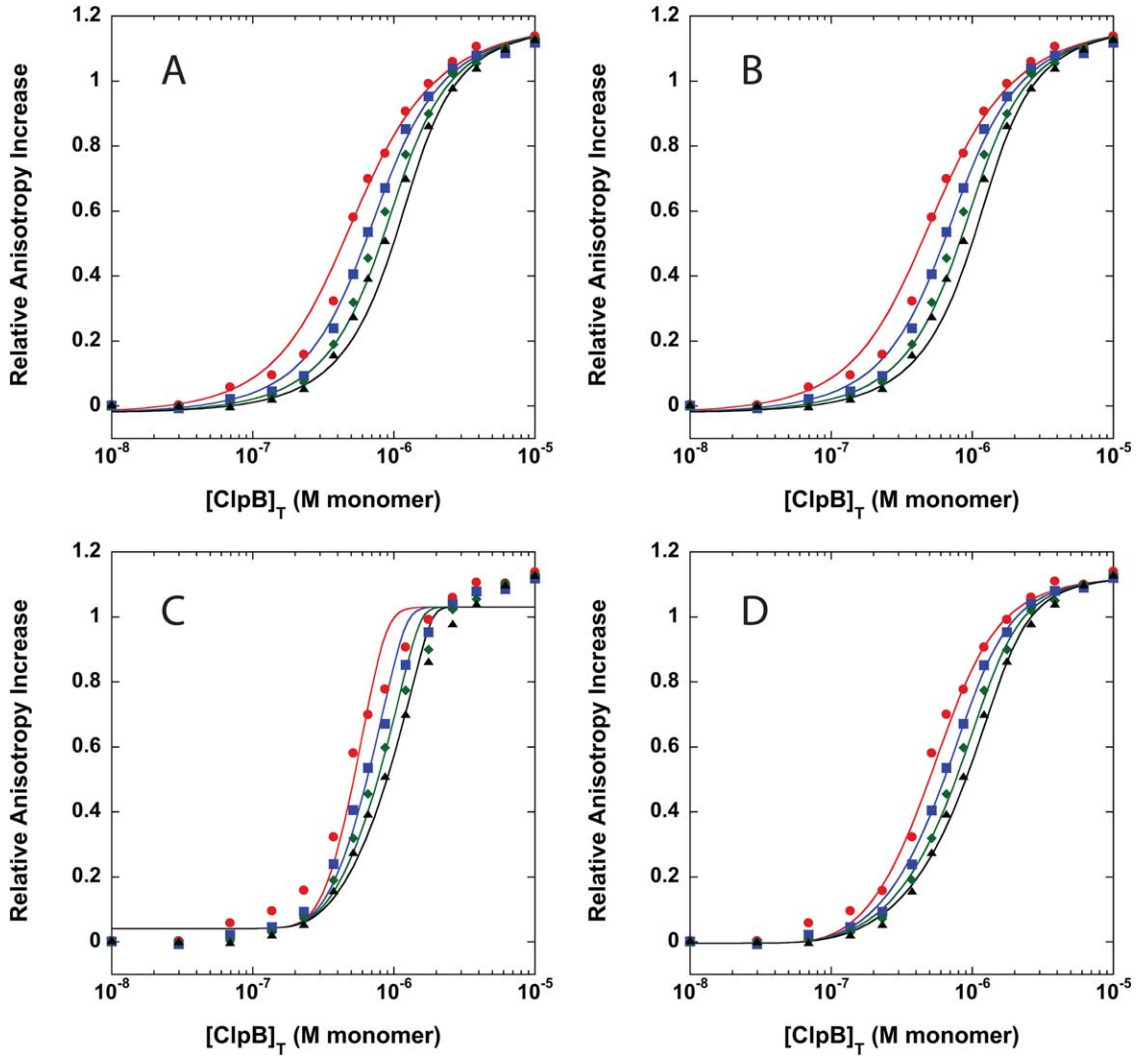

Figure 4

Global NLLS analysis of steady-state anisotropy titration data for Flu-αS1casein-102 titrated with ClpB in Figure 4E with (A) a 1:1 binding model [Eqs. (5, 13, and 14)], (B) an n-independent and identical sites model [Eqs. (5, 15, and 16)], (C) an infinite cooperative binding model [Eqs. (5, 16, and 17)], or (D) a hexamer binding model incorporating the dynamic equilibrium of ClpB assembly [Eqs. (5, 18, and 19)]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

global NLLS analysis with Eqs. (5, 16, and 17). However, the fits are too steep to describe the data (Fig. 4(C)). The data were also subjected to global NLLS analysis using the same infinite cooperative binding model but letting n float. The sum of squared deviations (SSD) value used to evaluate the goodness of the fits is 0.089887 (fit not shown), which represents an improvement compared to the fits (0.23775) when n was constrained to 6. Nevertheless, by inspection, the fit still exhibits substantial deviation from the experimental data.

The results of the data analysis above suggest that the binding resides somewhere between infinite cooperativity and no cooperativity. Thus, one way to describe the experimental observations is by deriving a relationship that couples the dynamic equilibrium between ClpB assembly to the ClpB binding process. It is important to note that the polypeptide substrate is being titrated with ClpB. Consequently, if ClpB resides in a dynamic equilibrium of monomers and hexamers, then the concentration of hexamers does not increase linearly with increasing ClpB monomer. In this model, the extent of binding, $\overline{X}$, can be calculated as described in Eq. 18,

$$\overline{X} = \frac{[\mathrm{ClpB}]_b}{[\mathrm{Peptide}]_T} = \frac{6[\mathrm{ClpB_6\text{-}Peptide}]}{[\mathrm{Peptide}]_T} = \frac{6K_6L_{6,\mathrm{app}}[\mathrm{ClpB}]_f^6}{1+K_6L_{6,\mathrm{app}}[\mathrm{ClpB}]_f^6} \quad (18)$$

where $[ClpB]_b$ is the concentration of ClpB monomers bound to the fluorescently modified polypeptide, which

Table II
Binding affinities for ClpB binding to various polypeptides

Name	Length (AA)	% composition of positively charged and aromatic residues	$K_{d,6}$ or $K_{d,6C}$ (nM)	$L_{6,app}$ (M^{-5})
αS1casein-127	127	17	16 ± 5	(7 ± 4) × 10^{33}
αS1casein-102	102	16	14 ± 4	(5 ± 2) × 10^{33}
N-Cys-50-SsrA	50	20	4 ± 2	(3 ± 1) × 10^{33}
N-Cys-50	50	24	3.0 ± 0.7	3 × 10^{33} [a]
N-Cys-40-SsrA	40	23	12 ± 3	(13 ± 8) × 10^{33}
SsrA	11	9	140 ± 20	3 × 10^{33} [a]
Peptide B1	21	33	1.7 ± 0.2	3 × 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 as the same as $L_{6,app}$ of N-Cys-50-SsrA. The uncertainty represents the standard deviation from the NLLS analysis.

is equal to [$ClpB_6$-$Peptide$], $[Peptide]_T$ is the total concentration of all bound and free polypeptides. $[ClpB]_f$ represents the concentration of free ClpB monomers that are not bound to polypeptide. The term that accounts for the dynamic equilibrium of ClpB assembly is $L_{6,app}$, which is the apparent self-assembly constant for ClpB hexamer and is defined in Eq. 7 in Materials and Methods. The conservation of mass equation is given by Eq. 19

$$[\mathrm{ClpB}]_T = [\mathrm{ClpB}]_f + 2L_{2,\mathrm{app}}[\mathrm{ClpB}]_f^2 + 4L_{4,\mathrm{app}}[\mathrm{ClpB}]_f^4 + 6L_{6,\mathrm{app}}[\mathrm{ClpB}]_f^6 + \overline{X}[\mathrm{Peptide}]_T \quad (19)$$

where $L_{2,app}$ and $L_{4,app}$ are the apparent self-assembly constants for ClpB dimers and tetramers, respectively [see Eqs. (8 and 9) for the definition of $L_{2,app}$ and $L_{4,app}$ in Materials and Methods]. We have determined the value for both $L_{2,app}$ and $L_{4,app}$ in the presence of 1 mM ATPγS from analytical ultracentrifugation experiments (J. Lin, manuscript in preparation). Thus, the value of $L_{2,app}$ and $L_{4,app}$ were constrained to 1.75 × 10^6 M^{-1} and 2.02 × 10^{20} M^{-3}, respectively. Whereas, in this analysis, $L_{6,app}$ was allowed to float as a fitting parameter.

The data in Figure 3(C) were subjected to global NLLS analysis using Eqs. (5, 18, and 19). The fits in Figure 4(D) clearly show that our data can be well described by the model that accounts for the dynamic equilibrium of ClpB assembly.

The BDF analysis suggested that there is a single ClpB hexamer per polypeptide, at saturation, for both αS1casein-127 and αS1casein-102. Thus, both sets of titration data were subjected to global NLLS analysis assuming that only a single ClpB hexamer binds and accounting for the dynamic equilibrium of ClpB assembly. From this analysis ClpB exhibits a $K_{d,6} = (16 \pm 5)$ nM and $L_{6,app} = (7 \pm 4) \times 10^{33}$ M^{-5} for αS1casein-127, where the parameters are within error of the parameters determined for ClpB binding to αS1casein-102, $K_{d,6} = (14 \pm 4)$ nM and $L_{6,app} = (5 \pm 2) \times 10^{33}$ M^{-5}(see Table II).

In this analysis we have assumed that only hexamers are binding. Control experiments performed in the absence of nucleotide or in the presence of nucleotide but in the absence of Mg^{2+} show no anisotropy change upon mixing ClpB with fluorescently modified polypeptide (data not shown). However, those experiments only show that the oligomers smaller than hexamers, which are highly populated under conditions lacking nucleotide, do not interact with the polypeptide. It does not rule out the possibility that oligomers smaller than hexamers that are bound by nucleotide could interact with the polypeptide.

Attempts to fit the titrations presented in Figure 4, including smaller oligomers, led to unconstrained parameters (fitting not shown). The observation that the fitting becomes unconstrained leads to the conclusion that there is not sufficient information in the titration curves to acquire information on the binding of oligomers smaller than hexamers, if present. Further, this likely indicates that if oligomers smaller than hexamers are binding then the observed signal must be dominated by the binding of hexamers. Nevertheless, a deeper examination into whether or not nucleotide ligated oligomers smaller than hexamers are binding is a topic for a future study.

Examination of ClpB binding to SsrA tagged polypeptide substrates

Both ClpX and ClpA bind specifically to the 11 amino acid tag at the c-terminus of polypeptide substrates.[42,43] However, Hinnerwisch *et al.* reported, as unpublished results, that ClpB does not recognize the SsrA tagged protein.[22] In a more recent review it was reported that ClpB does not interact with the SsrA tag citing Hinnerwisch and coworkers.[44] However, to our knowledge, the experimental data that support this conclusion have yet to be reported. Consequently, we tested whether ClpB can bind SsrA tagged polypeptides.

N-Cys-50-SsrA is a 50 aa polypeptide with the SsrA sequence at its C-terminus (see Table I). Similar to the αS1casein substrates, the substrate contains one cysteine residue at its N-terminus for the purposes of attaching a fluorescein dye. We have used this substrate to examine

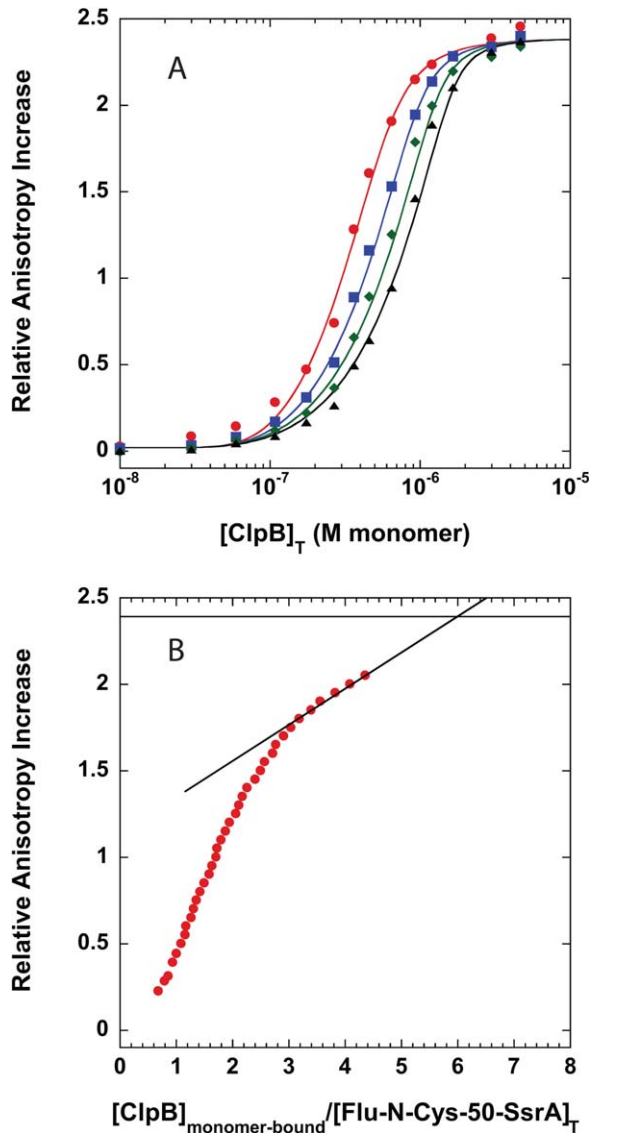

Figure 5

(A) Global NLLS analysis of steady-state anisotropy titration data collected at 64 nM (●), 128 nM (■), 192 nM (◆), and 255 nM (▲) Flu-N-Cys-50-SsrA titrated with ClpB using Eqs. (5, 18, and 19). (B) BDF analysis of steady-state anisotropy data in panel A. The black solid horizontal line represents the maximum observed signal increase for the four titrations in panel A of ~2.4.

ClpA binding and have reported a CD spectra showing that the substrate is unfolded.[26] To determine if ClpB can bind SsrA tagged polypeptide substrates, steady-state anisotropy titrations were performed by titrating 55, 110, 165, and 220 nM fluorescently modified N-Cys-50-SsrA (Flu-N-Cys-50-SsrA) with ClpB in the presence of 1 mM ATPγS as described in Materials and Methods and above. Similar to the titration isotherms that we have shown for αS1casein substrates, the steepness of the curves indicates that multiple ClpB monomers or oligomers are cooperatively binding to polypeptide substrates [see Fig. 5(A)].

To determine the number of ClpB monomers bound to the 50 amino acid substrate, the anisotropy isotherms for Flu-N-Cys-50-SsrA titrated with ClpB were subjected to the binding density function analysis. As seen in Figure 5(B), the relative anisotropy increase as a function of the extent of binding increases linearly up to ~1.5. By extrapolating to the observed maximum value in Figure 5(A) of ~2.4 Relative Anisotropy Increase, we were able to determine that the maximum stoichiometry for ClpB binding to Flu-N-Cys-50-SsrA is ~5.3 ClpB momoners per single polypeptide substrate, which likely represents hexameric ClpB bound to the substrate. The same BDF analysis was performed on the steady-state anisotropy titrations that were collected for N-Cys-40-SsrA (see Table II and Figure S2 in the supporting information). This result also suggests that there are six – seven ClpB monomers bound per single polypeptide. Taken together, our results from the BDF analyses are consistent with the hypothesis that a single ClpB hexamer is bound, at saturation, to each unstructured polypeptide substrate with length equal or less than 127 amino acids. However, we cannot rule out the possibility that smaller oligomers are also binding at sub-saturating concentration of ClpB. But, attempts to analyze the data using models that allow for binding of smaller oligomers did not improve the fits (analysis not shown). Consequently, we conclude that Eq. 18 represents a minimal model that will adequately describe the experimental observations.

The data in Figure 5(A) were also subjected to global NLLS analysis using Eqs. (5, 18, and 19). The binding affinity for Flu-N-Cys-50-SsrA is $K_6 = (3 \pm 2) \times 10^8$ M^{-1} ($K_{d,6} = (4 \pm 2)$ nM) and $L_{6,app} = (3 \pm 1) \times 10^{33}$ M^{-5}, where the parameters are defined in Eqs. 6 and 7. We have reported that the binding affinity of ClpA for the same substrate is $K_{d,6} = (4.7 \pm 0.8)$ nM,[45] which is within error of the value determined for ClpB ($K_{d,6} = (4 \pm 2)$ nM).

On the relatively short polypeptide substrates, like the 50 amino acid substrate, it is possible that ClpB binding could be affected by the presence of the dye. To test this possibility, competition steady-state anisotropy titrations were performed to examine the competition binding between Flu-N-Cys-50-SsrA and the identical substrate without the fluorophore, that is, non-labeled N-Cys-50-SsrA. The set of four anisotropy titrations were collected by titrating 110 nM Flu-N-Cys-50-SsrA in the presence of 50, 100, 150 and 200 nM of non-labeled N-Cys-50-SsrA by ClpB as described in Materials and Methods (see Figure S3A of the Supporting Information). As the concentration of non-labeled N-Cys-50-SsrA increased, the titration curve shifts to the right. This indicates that non-labeled N-Cys-50-SsrA is competing with Flu-N-Cys-50-SsrA for binding to ClpB.

To determine the binding affinity for non-labeled N-Cys-50-SsrA, we subjected the competition steady-state anisotropy data to global NLLS analysis using Eqs. (5,

10, 11, and 18) as previously reported.[26] In this analysis, the $K_{d,6}$ for Flu-N-Cys-50-SsrA was constrained to 4 nM, and $L_{6,app}$ was constrained to 3×10^{33} M^{-5} from the analysis of the titrations reported in Figure 5(A). The dissociation constant ($K_{d,6C}$) for ClpB hexamer binding to N-Cys-50-SsrA was found to be (2.0 ± 0.3) nM, which is within error with that of Flu-N-Cys-50-SsrA, $K_{d,6} = (4 \pm 2)$ nM. This result suggests that the fluorophore does not significantly affect the binding of ClpB to short polypeptide substrates. This observation also indicates that the fluorophore is not likely to be affecting the binding of ClpB to the αS1casein substrates.

The finding that ClpB binds to N-Cys-50-SsrA leads to the question; does ClpB specifically recognize SsrA or would ClpB simply bind to any unstructured polypeptides? To answer this question, competition steady-state anisotropy titrations were performed using N-Cys-50 (see Table I), which is an identical length substrate to N-Cys-50-SsrA but simply lacking the SsrA sequence. The binding affinity for N-Cys-50 is $K_{d,6C} = (3.0 \pm 0.7)$ nM (see Table II and Figure S3B of the supporting information), which is within error of the value ($K_{d,6} = (4 \pm 2)$ nM) for the same length peptide but containing the SsrA sequence (N-Cys-50-SsrA). This result suggests that, unlike ClpA, ClpB does not appear to specifically recognize the SsrA tag, that is, does not bind tighter to an otherwise identical substrate containing or lacking the SsrA sequence. However, the presence of the SsrA tag also does not preclude ClpB from binding.

Comparing the binding affinities among all the polypeptides ranging from 40 to 127 amino acids (Table II), all the $K_{d,6}$ values are comparable to each other and fall into the range of $\sim 3 - 16$ nM regardless of the source of these polypeptides. Consequently, the substrates tested so far indicate that there is little specificity for ClpB binding to various unstructured polypeptides. However, relative to the $\sim 10 - 20$ μM concentration of ClpB in living cells the binding affinity is relatively tight.[46]

In contrast to ClpB, we have reported that ClpA binds to the 11-amino acid SsrA sequence with $K_{d,6} = (200 \pm 30)$ nM. However, the value of $K_{d,6}$ decreases to $\sim 3 - 5$ nM when the SsrA sequence is incorporated into a longer unstructured substrate.[45] To examine the binding affinity between ClpB and SsrA, competition anisotropy titration data were collected by titrating 100 nM Flu-N-Cys-50-SsrA in the presence of 50, 200, 350 and 500 nM non-labeled SsrA by ClpB as described in Materials and Methods (see Figure S3D of the Supporting Information). After global NLLS analysis, the binding affinity for the SsrA sequence (11 amino acids) was found to be $K_{d,6} = (140 \pm 20)$ nM (Table II), which is much weaker compared to the longer polypeptide substrates examined here.

The observation that the ClpB binding affinity for the eleven amino acid SsrA sequence is much weaker than the 50 amino acid sequence containing SsrA is identical to what we have reported[26] for ClpA, and could be explained by two possibilities. First, similar to the conclusion we made for ClpA, 11 amino acids may not provide sufficient contacts for binding of one ClpB hexamer. Second, the reduced percentage composition of positively charged and aromatic residues in the SsrA sequence (9 %, see Table II) could also be why ClpB exhibits weaker binding to the 11-amino acid SsrA sequence compared to other longer substrates examined here. However, these two possible explanations are not mutually exclusive.

ClpB binding to polypeptide B1

Schlieker and coworkers[17] identified a 21-amino acid sequence, Peptide B1, which they concluded can be tightly bound by ClpB. Thirty-three percent of amino acids that make up Peptide B1 are positively charged and aromatic. They reported a binding affinity for wild-type ClpB binding to Peptide B1 as $K_d < 80$ nM. Also reported was a value of $K_d = (23 \pm 2)$ nM for binding a ClpB mutant with mutations in the Walker B motif of both AAA+ domains, termed ClpB-B1/2A to Peptide B1. Both values were obtained under the assumption that all ClpB is in the hexameric state.

We examined the binding affinity between wild-type ClpB and Peptide B1 accounting for the dynamic equilibrium of ClpB assembly. To do this, competition anisotropy titration isotherms were collected by titrating 100 nM Flu-N-Cys-50-SsrA in the presence of 50, 100, 150 and 200 nM of non-labeled Peptide B1 with ClpB as described in Materials and Methods. Next, the titration data were subjected to global NLLS analysis accounting for the dynamic equilibrium of ClpB assembly (see Figure S3C of the Supporting Information). A binding affinity of $K_{d,6C} = (1.7 \pm 0.2)$ nM was determined for Peptide B1 (Table II).

The other polypeptides with lengths of $40 - 127$ amino acids that we tested contain ~ 20 % charged and aromatic residues compared to that of Peptide B1, which contains ~ 33 % (see Table II). The binding affinity for these polypeptides are in a range of $\sim 3 - 16$ nM, which is weaker, but still comparable to that of Peptide B1 ($K_{d,6C} = (1.7 \pm 0.2)$ nM). This result suggests that ClpB exhibits modest substrate specificity for Peptide B1.

DISCUSSION

In this study we quantitatively investigated the binding between ClpB and various unstructured polypeptides using steady-state anisotropy titration experiments. Every set of four titrations for each substrate was subjected to global nonlinear least squares analysis accounting for the dynamic equilibrium of the ClpB assembly reaction. Compared to single curve analysis, global analysis provides more constraints and improves the precision of the fitting parameters, especially for highly correlated parameters.[47] Thus, we are able to report, with higher precision, the values of the

hexameric ClpB binding affinity constant, $K_{d,6}$, and the apparent ClpB assembly constant, $L_{6,app}$, as we have recently done for ClpA.[26] Moreover, the information on the presence of a dynamic equilibrium exists within how the titration curves shift to higher ligand concentration as the total macromolecule concentration is increased.

Incorporating the dynamic equilibrium of ClpB assembly into the analysis of polypeptide binding

ClpB resides in a dynamic equilibrium of oligomers. Previous studies by others[15,16] and analytical ultracentrifugation studies from our lab (J. Lin, manuscript in preparation) have shown that ClpB does not exist in solution only as hexamers and the species distributions of ClpB oligomers depends on many factors. These factors include concentration of ClpB, nucleotide concentration and type, salt concentration and type, among other buffer components. The experimental condition in our steady-state anisotropy titrations are 200 mM NaCl, 10 mM $MgCl_2$, and 1 mM ATPγS with the total ClpB monomer concentration varying between 10 nM and 10 μM.

Equations 18 and 19 describe hexameric ClpB binding to polypeptide accounting for the dynamic equilibrium of the assembly reaction. It is not possible to express Eq. 18 as a function of the total ClpB monomer concentration because the conservation of mass equation, given by Eq. 19, is a sixth order polynomial in the free ClpB monomer concentration. Thus, in order to examine binding data for ClpB, the system of equations given by Eqs. (18 and 19) must be solved numerically and implicitly for the free ClpB monomer concentration. Indeed, if ClpB formed only hexamers and did not dissociate over the concentration range of the titrations, then a substantially simpler fitting strategy could be invoked.

Previously reported titrations to examine ClpB binding to polypeptide substrates assumed that all ClpB is in the hexameric state.[17] To test this assumption, we subjected our titration data to global NLLS analysis with a model that assumes that all ClpB reside in the hexameric state. However, this model failed to simultaneously describe all four titration curves [see Fig. 4(A)]. Thus, it is unlikely that the total ClpB monomer concentration is entirely in the hexameric state. In contrast, the titration data can be well described when we account for the dynamic equilibrium of ClpB assembly [Fig. 4(D)].

Schlieker *et al.* reported an estimation of the binding affinity, K_d, between wild-type ClpB hexamer and Peptide B1, a substrate they identified, as being less than 80 nM.[17] They also reported a value of $K_d = (23 \pm 2)$ nM for binding to the same substrate by a ClpB mutant with mutations in the Walker B motif of both AAA+ domains, termed ClpB-B1/2A. In their binding study, the concentration of ClpB hexamers was obtained by dividing the total ClpB monomer concentration by six, which assumes only hexamers reside in solution.

We also examined the binding of ClpB to Peptide B1. After global NLLS analysis incorporating the assembly of ClpB, we determined a $K_{d,6} = (1.7 \pm 0.2)$ nM for the wild-type ClpB binding to Peptide B1 in the presence of ATPγS. The resulting binding affinity is more than 10-fold tighter than the binding affinity ($K_{d,6} = (23 \pm 2)$ nM) reported by Schlieker *et al.* for ClpB-B1/2A in the presence of hydrolysable ATP and more than 40-fold tighter than for ClpB wild-type.

Schlieker *et al.* pointed out that there are three criteria for characterizing the binding of ClpB to polypeptide substrates.[17] The first criterion is that ClpB binds to substrates in an ATP-dependent manor. The second is that the oligomeric state of ClpB can be stabilized by binding to the substrate. The third is that the substrate can stimulate ClpB ATPase activity. These three criteria can be quantitatively summed up in the definition of $L_{6,app}$ in Eq. 20

$$L_{6,app} = \frac{\{ClpB_6\}}{\{ClpB\}^6} = \frac{[ClpB_6]}{[ClpB]^6}\frac{P_6}{(P_1)^6} = L_{6,0}\frac{P_6}{(P_1)^6} \tag{20}$$

where P_6 is the partition function for all of the hexameric nucleotide ligation states normalized to the unligated hexamer given by Eq. 21

$$P_6 = \frac{[ClpB_6]}{[ClpB_6]} + \frac{[ClpB_6\text{-}ATP_1]}{[ClpB_6]} + \frac{[ClpB_6\text{-}ATP_2]}{[ClpB_6]} + \ldots + \frac{[ClpB_6\text{-}ATP_{i\text{-}1}]}{[ClpB_6]} + \frac{[ClpB_6\text{-}ATP_i]}{[ClpB_6]} \tag{21}$$

where the subscript i represents the maximum number of ATP molecules that can bind to ClpB hexamers, which could be up to 12, P_1 is the partition function for all of the monomeric nucleotide ligation states normalized to the unligated monomer given by Eq. 22

$$P_1 = \frac{[ClpB]}{[ClpB]} + \frac{[ClpB\text{-}ATP_1]}{[ClpB]} + \frac{[ClpB\text{-}ATP_2]}{[ClpB]} \tag{22}$$

Since the monomer of ClpB has two binding sites the maximum number of nucleotides bound is two. However, it is not known if the monomer binds nucleotide or if both nucleotide binding sites in the monomer are bound. $L_{6,0}$ is defined by the equilibrium given by Eq. 23, and is given by Eq. 24,

$$6ClpB \overset{L_{6,0}}{\rightleftharpoons} ClpB_6 \tag{23}$$

$$L_{6,0} = \frac{[ClpB_6]}{[ClpB]^6} \tag{24}$$

which represents the assembly of hexamers in the absence of nucleotide, where zero in the subscript represents no nucleotide bound.

$L_{6,app}$ given by Eq. 20 reveals that the concentration of ClpB hexamers present in solution is governed by the nucleotide ligation state of the hexamer. This is because $L_{6,0}$ is the hexamerization equilibrium constant in the absence of nucleotide and does not have any dependence on nucleotide concentration. Consequently, the only ways $L_{6,app}$ can increase and thus increase the concentration of hexamers present in solution are for P_6 to increase or P_1 to decrease. Both of these partition functions are functions of the nucleotide concentration.

Thus, the first criterion that the binding to polypeptide is ATP dependent is evidence that not all of the ClpB is in the hexameric state. If all of the ClpB were in the hexameric state the binding would be observed to be independent of nucleotide. The second criterion that polypeptide binding stabilizes the oligomeric state of ClpB is simply a statement of reciprocity. If the concentration of hexamers present and available to bind polypeptide is increased with nucleotide binding then so must the polypeptide binding be observed to increase. Finally, the ATPase activity will be observed to be stimulated by polypeptide binding because the binding of nucleotide has been enhanced in the presence of polypeptide.

In order to quantitatively examine binding, assembly, or catalysis, one must have a precise determination of the concentration of the active form of the protein. In the case of both ClpA and ClpB, the active form of the protein is hexameric. However, we have shown for ClpA,[48] Del Castillo *et al.*[16] has shown for ClpB, and we have confirmed, that these enzymes reside in a dynamic equilibrium and cannot be assumed to be 100 % hexameric under all solution conditions and total protein concentrations. Rather, in order to predict the concentration of hexamers in solution one must have precise determinations of the equilibrium constants defining the dynamic equilibrium.

According to the definition, the concentration of both ClpB and nucleotide affect the value of $L_{6,app}$, and $L_{6,app}$ defines the concentration of ClpB hexamers bound with nucleotides, which further affects the apparent value of $K_{d,6}$. Thus, the apparent binding specificity of ClpB is not only determined by the binding affinity, $K_{d,6}$, but is also impacted by the apparent ClpB hexamer assembly constant, $L_{6,app}$.

We simulated the species distribution of ClpB oligomers using the determined $L_{2,app} = 1.75 \times 10^6\ M^{-1}$, $L_{4,app} = 2.02 \times 10^{20}\ M^{-3}$, and $L_{6,app} = 6.58 \times 10^{34}\ M^{-5}$ from analytical ultracentrifugation experiments where ClpB was incubating in the presence of 1 mM ATPγS (J. Lin, manuscript in preparation). Figure 6 shows the species fraction as a function of the total ClpB monomer concentration. As seen in Figure 6, the monomeric ClpB concentration decreases with increasing total ClpB concentration shown in red. Dimers and tetramers are sparsely populated shown in blue and green, respectively.

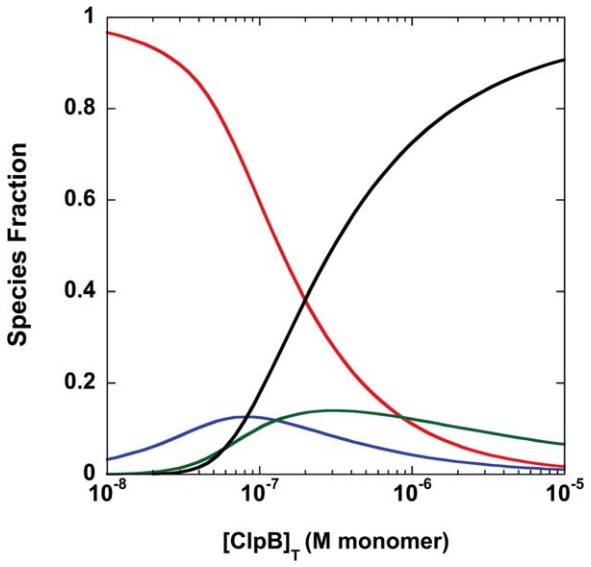

Figure 6

Simulation of species distribution of ClpB monomer (red), dimer (blue), tetramer (green) and hexamer (black) in the presence of 1 mM ATPγS. $L_{2,app}$, $L_{4,app}$ and $L_{6,app}$ were constrained to the value of $1.75 \times 10^6\ M^{-1}$, $2.02 \times 10^{20}\ M^{-3}$, $6.58 \times 10^{34}\ M^{-5}$, respectively.

The concentration of ClpB hexamers are increasing with increasing total ClpB concentration shown in black. The simulation in Figure 6 shows that the monomeric ClpB is a predominant form in the low concentration range of the total ClpB monomer (10 – 100 nM), and ClpB hexamers become the predominant species above a concentration of 1 μM.

The normal cellular concentration of ClpB has been reported to be ~9 μM and 19 μM in monomer units at 30 and 42°C, respectively.[46] At these concentrations the majority of ClpB is in the hexameric state. However, most in vitro experiments are carried out at ClpB concentrations lower than 9 μM monomer where a distribution of oligomers will be present.

Stoichiometry for ClpB binding to polypeptide

Our titration data were subjected to the BDF analysis as described.[28,33–35,49] This model-independent method allows us to determine the estimated maximum binding stoichiometry at saturating ClpB concentrations. For the polypeptide substrates that are ≤ 127 amino acids, our results suggest that there are about six ClpB monomers bound per single polypeptide. This is consistent with a single ClpB hexamer binding to a polypeptide substrate. Only assuming that ClpB hexamers bind doesn't give rise to the observed steepness of the titration curves. Rather, what gives rise to the steepness of the curve is the dynamic equilibrium of ClpB assembly and the fact that the concentration of hexameric ClpB varies

at each titration point. Interestingly, BDF analysis shows that more than six ClpB monomers (around 12 monomers) bind to longer substrates examined here (152 and 177 amino acids).

Nonspecific binding to unstructured polypeptides

We have previously reported CD spectra for the polypeptide substrates examined here, and these CD spectra are consistent with random coils.[28] To begin to examine the binding specificity for ClpB binding to these unstructured polypeptide substrates, we quantitatively investigated the binding affinities, K_d, and the linkage to assembly, $L_{6,app}$. Here we define the substrate specificity as a measurement of the relative binding affinity. Consequently, the higher the specificity, the smaller the dissociation equilibrium constant, K_d. ClpB exhibits a $K_{d,6} = \sim 15$ nM for αS1casein truncations (αS1casein102 and αS1casein127) (see Table II). The $K_{d,6}$ values for peptides derived from TitinI27-SsrA[50] (N-Cys-40-SsrA and N-Cys-50-SsrA) are in a range of about 3 – 12 nM. All of these binding constants are comparable to each other indicating that ClpB has little discernible preference in binding to the unstructured polypeptides examined here.

It has been reported that ClpB favors binding to polypeptides that are enriched with positively charged and aromatic amino acid residues. In that study the authors reported that ClpB can bind tightly to a 21-amino acid polypeptide (Peptide B1) containing 33 % positively charged and aromatic residues.[17] Our study shows that ClpB exhibits a $K_{d,6} = (1.7 \pm 0.2)$ nM for Peptide B1. This value is ~2 – 8-fold smaller than the binding affinities for other polypeptides that we have examined here. This result indicates that ClpB exhibits a modest specificity for Peptide B1 compared to other substrates we examined, which are made up of 16 - 24 % of both positively charged and aromatic residues (Table II). However, the results presented here do not represent a thorough examination of the dependence of the binding affinity on the sequenced composition. This remains a topic for future studies.

Binding to the SsrA sequence

Both Hinnerwisch *et al.* and Zolkiewski *et al.* reported that ClpB does not recognize or interact with SsrA tagged proteins.[22,44] However, to our knowledge, the experimental data that support this conclusion has yet to be reported. Thus, we tested whether ClpB can recognize SsrA tagged polypeptides by determining the binding affinity for substrates containing the SsrA sequence. The ClpB binding affinity for the 50-amino acid substrate with the SsrA sequence at the C-terminus was found to be $K_{d,6} = (4 \pm 2)$ nM, which is within error of the ($K_{d,6C} = (3.0 \pm 0.7)$ nM) for the 50-amino acid polypeptide lacking the SsrA tag. This result suggests that ClpB can bind to both of these substrates, but it is not clear if ClpB is recognizing the SsrA tag.

To further test whether ClpB can recognize SsrA, we examined the binding of ClpB to the minimum 11-amino acid SsrA sequence. Interestingly, the binding affinity for ClpB binding to the 11 amino acid SsrA sequence is (140 ± 20) nM, which is ~10 – 50 fold weaker than all other unstructured polypeptides that we tested here. One possibility for the reduced binding affinity is that the SsrA sequence contains about 9 % of both positively charged and aromatic residues, which is less than that of other polypeptides examined here (16 – 33 %). According to Schlieker's conclusion that ClpB prefers to bind to positively charged and aromatic amino acid residues, fewer positively charged and aromatic residues in the SsrA sequence could disfavor binding of ClpB. To resolve this question, there remains a need to systematically compare the binding affinities of ClpB to polypeptides with identical length but changing the percentage of positively charged and aromatic residues.

An alternative explanation for the observed weaker binding affinity for the minimal length SsrA sequence is that the length of the 11-amino acid SsrA sequence is too short. It has been well established that ClpA specifically recognizes and binds to the SsrA sequence.[24,42] Also, we recently reported that when the SsrA sequence is incorporated into unstructured polypeptides with a total length of 50 amino acids, ClpA hexamers will bind with a $K_{d,6} = (4.7 \pm 0.8)$ nM. However, ClpA exhibits a $K_{d,6} = (200 \pm 30)$ nM for the 11-amino acid SsrA sequence, which is ~20-fold weaker than binding to the SsrA tagged 50-amino acid substrate.[28] Since ClpA can specifically recognize SsrA, the most likely explanation for the observed weaker binding is that the 11-amino acid SsrA sequence does not provide all of the necessary contacts for maximum binding affinity.

ClpB and ClpA are homologous proteins and both belong to the Class I Clp/Hsp100 chaperone family. Both enzymes have one N-terminal domain and two tandem AAA+ domains, termed Domain 1 (D1) and Domain 2 (D2), respectively. D1 and D2 are both Walker-type nucleotide binding domains with conserved Walker A and Walker B motifs. In the primary structure, there is a conserved region between the Walker A and Walker B motifs termed the pore loop that is crucial for substrate binding.[17,18,20,21] When ClpB or ClpA forms the ring-shaped hexamer, the D1 pore loop and D2 pore loop are facing into the axial channel of the hexamer.[18,19] It has been shown that polypeptide substrates interact with these D1 and D2 pore loops in the axial channel of ClpA.[22] Since ClpB has the same conserved pore loops as ClpA, it is possible that ClpB also uses the pore loops to interact with polypeptide substrates during protein disaggregation.

Schlieker *et al.*[17] and Weibezahn *et al.*[21] used cross-linking techniques to identify ClpB-peptide binding sites. A cross-linking product of ClpB interacting with Peptide B1 through the D1 pore loop was observed by Schlieker *et al.* Later, Weibezahn *et al.* detected cross-linking products of Peptide B1 linking to ClpB's D2 pore loop. Their results suggest that both D1 and D2 pore loops directly interact with Peptide B1. However, it is still not clear whether Peptide B1 interacts with both the D1 and D2 loops simultaneously.

The length of the axial channel formed by the hexameric ring is 90 Å for ClpB[51] and 87 Å for ClpA.[13] The estimated distance between the D1 pore loop and D2 pore loop for both ClpB and ClpA hexamers is about 40 – 50 Å. SsrA has been shown to interact with the D2 loop of ClpA.[22] However, the 11-amino acid SsrA sequence only spans up to 35 Å,[22] which indicates SsrA may be too short to make contacts with both the D1 and D2 pore loops simultaneously. On the other hand, Peptide B1, containing 21 amino acids, is about twice as long as the SsrA sequence, which makes it possible for Peptide B1 to make simultaneous contact with both the D1 and D2 pore loops.

In summary, ClpB and ClpA have a number of structural similarities. The same trend of binding to SsrA and the SsrA tagged 50-amino acid polypeptide substrate was observed for ClpB as was for ClpA. This could mean that SsrA interacts with ClpB in the same way that ClpA does. Thus, we hypothesize that ClpB, like ClpA, requires a minimum length that resides between 11 and 21 amino acids of unstructured polypeptide for optimal binding to both the D1 and D2 pore loops.

CONCLUSION

ClpB works as a disaggregation machine to protect cells from the accumulation of aggregated proteins. Our knowledge of how ClpB recognizes aggregates is still limited. If there are no specific sequences or tags on protein aggregates that ClpB interacts with, then the question remains as to what ClpB recognizes and binds to in the protein aggregates. Disordered polypeptide tails or loops are likely to be excluded from protein aggregates. Here we conclude that hexameric ClpB binds to a variety of unstructured polypeptides with a relatively tight binding affinity in the range of ~3 – 16 nM. This observation may indicate that ClpB binds to a variety of exposed tails and loops. Furthermore, we observed that multiple ClpB hexamers can bind to long ($\geq$ 152 amino acids) unstructured polypeptides. This observation may indicate that multiple ClpB hexamers may cooperate to disrupt protein aggregates.

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