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Interaction of Substrate-Mimicking Peptides with the AAA+ ATPase ClpB from *Escherichia coli*

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Abstract

A molecular chaperone ClpB disaggregates and reactivates aggregated proteins in cooperation with DnaK, DnaJ, and GrpE. Within a cellular environment, ClpB must distinguish between properly folded and aggregated proteins by recognizing specific physical and/or chemical surface properties of the aggregates. However, the molecular mechanism of substrate binding to ClpB is poorly understood. We hypothesized that ClpB recognizes those polypeptide segments that promote protein aggregation because they are likely present at the surface of growing aggregates. We used an algorithm TANGO (Fernandez-Escamilla et al., *Nat. Biotech.* 2004, 22, 1302) to predict the aggregation-prone segments within the model ClpB-binding peptides and investigated interactions of the FITC-labeled peptides with ClpB using fluorescence anisotropy. We found that ClpB binds the substrate-mimicking peptides with positive cooperativity, which is consistent with an allosteric linkage between substrate binding and ClpB oligomerization. The apparent affinity towards ClpB for peptides displaying different predicted aggregation propensities correlates with the peptide length. However, discrete aggregation-prone segments within the peptides are neither sufficient nor necessary for efficient interaction with ClpB. Our results suggest that the substrate recognition mechanism of ClpB may rely on global surface properties of aggregated proteins rather than on local sequence motifs.

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Keywords

molecular chaperone; ClpB; AAA+ ATPase; protein-peptide interactions; fluorescence anisotropy

ClpB is a bacterial member of the Hsp100 family of ATP-dependent molecular chaperones that disaggregate and reactivate aggregated proteins in cooperation with Hsp70 and Hsp40 [1-3]. Hsp100s are found in bacteria, protozoa, fungi, and plants, but are absent in metazoan proteomes. Hsp100s belong to the superfamily of ATPases associated with different activities (AAA+) [4]. In solution, ClpB forms a dynamic ensemble of homo-oligomers with hexamers predominating in the presence of nucleotides [5-7]. Hexameric ClpB forms a cylinder-shaped particle with a narrow axial channel [8]. The cylindrical hexamer displays a significant conformational plasticity, as evidenced by its propensity to form semi-open spiral-like configurations [9]. The ClpB-mediated aggregate reactivation is linked to extraction of single polypeptides from aggregated particles and their forced unfolding during translocation through the central channel in the hexamer [10]. A ratchet-like pulling mechanism of substrate translocation is supported by the conformational plasticity of the ClpB hexamer [11, 12]. Extracted polypeptides are released from the ClpB channel after either partial translocation or complete unfolding [13-15].

While the substrate-translocation principle of the ClpB-mediated protein disaggregation has been well documented and is indeed consistent with the mechanism of many AAA+ ATPases, an upstream step of substrate recognition and binding to ClpB is poorly understood. In cells, the Hsp70/Hsp40 chaperones (DnaK/DnaJ/GrpE in bacteria) assist in targeting ClpB to the sites of aggregate accumulation [16]. DnaK also stimulates the substrate extraction *via* a direct interaction with the coiled-coil domain of ClpB [17-20].

An essential role of DnaK notwithstanding, ClpB itself is capable of recognizing aggregated proteins and forming stable complexes with substrates in its ATP-bound conformation [21-24]. An ATP-hydrolysis deficient “substrate-trapping” ClpB variant became a useful tool for investigation of ClpB-substrate interactions [21]. Multiple evidence indicates that the ClpB-substrate complexes formed in the presence of ATP and absence of DnaK represent “on-pathway” early intermediates of the disaggregation reaction. First, the DnaK-independent ClpB-aggregate interaction occurs in the ATP-bound, but not ADP-bound or nucleotide-free state, which is consistent with the well documented AAA+ mechanism of substrate recognition [21, 24, 25]. Second, in the ATP-state, ClpB discriminates between the residue types in substrates [22] and between aggregates and native proteins [26]. Third, the residue substitutions in ClpB, which inhibit the DnaK-independent substrate binding, also inhibit the disaggregation [23, 24]. Fourth, the DnaK-independent substrate binding is consistent with the co-chaperone-independent reactivation of some aggregates by ClpB [27, 28] and with a standalone disaggregase activity of the *Pseudomonas aeruginosa* ClpB variant, ClpG [29].

We hypothesized that ClpB recognizes those polypeptide segments that promote protein aggregation because they are likely present at the surface of growing aggregates and we decided to search for such sequence segments within the known peptide-based model substrates of ClpB [22]. We used an aggregation-propensity prediction algorithm to identify

the aggregation-prone segments in the model ClpB substrates and we investigated a correlation between the aggregation-propensity and the ClpB-binding affinity. We discovered that discrete aggregation-prone segments within the model ClpB substrates are neither sufficient nor necessary for strong interaction with ClpB.

Materials and Methods

Peptides and proteins

The peptides listed in Table I were purchased from Peptide 2.0 (Chantilly, Virginia). The peptides were N-terminally labeled with FITC and C-terminally amidated by the manufacturer. Peptide concentration was determined spectrophotometrically using the FITC molar extinction coefficient $77,000 \text{ cm}^{-1}\text{M}^{-1}$ at 494 nm. The ATP-hydrolysis deficient substrate-trapping ClpB variant (E279Q/E678Q) was produced as described before [23]. ClpB concentration was measured using the calculated extinction coefficient $0.38 \text{ cm}^2/\text{mg}$ [30].

Aggregation-propensity prediction

The TANGO algorithm (<http://tango.crg.es>) [31, 32] was used to compute the aggregation-propensity profiles for the peptide sequences (see Fig. 1).

Peptide aggregation determination

The turbidity of peptide solutions was measured using a BioTek Synergy H1 plate reader. A 96-well plate with 85 μl per well of buffer A (50 mM Tris-HCl, pH 7.5, 200 mM KCl, 20 mM MgCl_2) was equilibrated at 40 °C in the reader chamber. After 10 minutes of pre-incubation, 15 μl of 1 mM peptide solution in DMSO was added to the wells with 15 μl of DMSO used as a control. Immediately after insertion into the reader, the plate was shaken inside the device for 10 s and the absorbance time course at 550 nm was recorded (see Fig. 2).

ClpB-peptide interactions

Fluorescence anisotropy measurements were performed using a PerkinElmer LS55 fluorescence spectrometer equipped with automatic polarizers. The FITC-labeled peptides were dissolved in buffer A supplemented with 2 mM ATP or ADP. The peptide concentration was 18 nM (Figs. 3A, 4, 5) or 80 nM (Fig. 3B). The excitation and emission wavelengths were 494 nm and 521 nm, respectively. Before addition of ClpB, the peptide solutions were equilibrated at room temperature for 10 minutes and their fluorescence anisotropy was recorded. Next, the substrate-trapping ClpB variant (E279Q/E678Q) was added in a step-wise manner to the cuvette with manual mixing. After each addition of ClpB, the samples were incubated for 2 min at room temperature, the anisotropy readings were collected during the following 2 min and averaged. Each titration was performed twice with the average anisotropy readings and their standard deviations calculated using the GraphPad Prism software. The change of anisotropy, Δr , was obtained by subtracting the initial anisotropy reading for the ClpB-free peptides from the anisotropy measured during the titrations with ClpB. GraphPad Prism was used for nonlinear least-squares fitting of the model assuming cooperative binding of ClpB to the peptides according to the equation: $r =$

($r_{\max} [\text{ClpB}]^h / (K_d^h + [\text{ClpB}]^h)$), where r is the observed change in anisotropy upon a ClpB addition, $r_{\max}$ is the fitting parameter corresponding to an anisotropy change at saturation, K_d is the apparent dissociation constant for the peptide-ClpB complex, and h is the Hill coefficient describing an extent of cooperativity in binding (see Table II).

Results

The 20-residue peptides B1 and B2 (see Table I) are enriched in Lys and Arg and have been shown to mimic multiple hallmark features of the ClpB-aggregate interactions [22]. We used the TANGO algorithm [31, 32] to test if the peptides B1 and B2 contain discrete sequence segments that could promote beta-sheet driven aggregation. As shown in Fig. 1A, B, the peptide B1 contains one, and B2 two aggregation-prone segments. Moreover, the predicted aggregation scores for B2 are higher than for B1. Notably, the predicted aggregation-prone segments in B1 and B2 are flanked by positively charged residues, a tendency found among the aggregation-promoting sequences across multiple proteomes [33].

We scrambled the sequences of B1 and B2 to modify their predicted aggregation profiles. First, we obtained the sequences B1N and B2N (see Table I), for which the TANGO aggregation scores were insignificant (data not shown). Next, we obtained the sequence B2A, for which the aggregation scores were significantly higher than for B2 (Fig. 1C) and the sequence B2B with only one aggregation-prone segment and lower aggregation scores than for B2 (Fig. 1D). Thus, we designed a number of sequences with the same amino-acid composition as B1 and B2, but with a range of different aggregation propensities, according to the TANGO predictions. The peptides B1, B1N, B2, B2N, B2A, and B2B have been synthesized and labeled with FITC at their N-termini. We also obtained the FITC-labeled peptides B1T, B2T1, and B2T2 (see Table I), which correspond to the aggregation-prone segments within B1 and B2, including their flanking basic residues.

We investigated if the calculated aggregation propensity of the peptides correlates with their solubility in aqueous solutions. All the labeled peptides were soluble in aqueous buffers at sub-micromolar concentrations that were used in the binding experiments with ClpB (see below). However, we found that the peptides B2 and B2A caused a significant time-dependent increase in solution turbidity when diluted to 150 μM at 40 $^{\circ}\text{C}$. Notably, the turbidity increase for B2A was considerably faster than for its parent peptide B2 while those of B2N and B2B were insignificant (Fig. 2), which shows a correlation between the TANGO predictions of the peptide aggregation propensity (see Fig. 1) and their behavior in solution. We did not detect a significant turbidity in the solutions of B1 and B1N, in agreement with their lower predicted aggregation scores, as compared to B2 and B2A. Altogether, the results in Fig. 2 validate the aggregation-propensity ranking of the peptides based on the TANGO algorithm (see Fig. 1).

We used fluorescence anisotropy to investigate the peptide interactions with ClpB. Binding of a FITC-labeled peptide to hexameric ClpB (~570 kDa) significantly decelerates the rotational diffusion of the fluorescein dye and produces an increase in fluorescence anisotropy. Indeed, a dose-dependent increase in fluorescence anisotropy was observed during titration of B1 and B2 with the substrate-trapping ClpB variant in the presence of

ATP, but not when ADP was present (Fig. 3A). ClpB with ATP did not affect the anisotropy of a free fluorescein, which indicates that the interaction with ClpB is mediated by the peptide core and not by the label moiety (Fig. 3A). The ATP-dependent binding of B1 and B2 confirms that ClpB recognizes these peptides as pseudo-substrates, as postulated before [22].

To estimate the binding stoichiometry, we conducted titrations of the peptides with ClpB using a ~4-fold higher peptide concentration, as compared to the conditions of Fig. 3A. The fluorescence anisotropy increases for B1 and B2 reached an apparent saturation at ~1:1 ratio of the peptide/ClpB hexamer concentrations (Fig. 3B). This result indicates a single peptide binding site within the ClpB hexamer and is consistent with the substrate binding in the axial channel formed at the center of the hexamer, as determined before [22].

A sigmoidal shape of the binding isotherms for B1 and B2 in the presence of ATP (see Fig. 3A), suggested a positive cooperativity in binding to ClpB. Fitting a cooperative binding model to the data in Fig. 3A gave the Hill coefficient of ~2.5 for B1 and B2 (see Table II). The apparent dissociation constant for B2 was approximately three-fold lower than for B1. By using the N-terminally truncated ClpB, we confirmed a previous result [22] that the N-terminal domain of ClpB, which supports binding to large aggregates [23], is not required for interactions with B1 and B2 (data not shown).

Next, we asked if the predicted aggregation-prone segments in B1 and B2 (see Fig. 1) are sufficient for producing a ClpB-binding capability. As shown in Fig. 4 and Table II, ClpB showed much weaker affinity towards the shorter peptides B1T, B2T1, and B2T2 than their parent peptides B1 and B2. The binding of B1T, B2T1, and B2T2 to ClpB remained ATP-dependent (data not shown), which indicates a specific recognition of the TANGO-predicted peptide segments by ClpB, but such recognition is insufficient to account for the binding affinity of B1 and B2.

We also asked if the predicted aggregation-prone segments in B1 and B2 are necessary for recognition by ClpB. As shown in Fig. 5A, B and Table II, the peptides B1N and B2N, which did not contain any predicted aggregating segments, showed a strong binding competency towards ClpB, equal to or exceeding that of the parent peptides B1 and B2. Moreover, ClpB interacted with the peptides B2A and B2B with a similar affinity (Fig. 5B, Table II), in spite of a significant difference between their predicted aggregation propensity profiles (see Fig. 1) and aqueous solubility (see Fig. 2).

Discussion

TANGO is one of the algorithms for assessing aggregation propensity of polypeptides from their amino acid sequence [31, 32, 34]. TANGO accurately predicted aggregation of many peptide sequences, including the Alzheimer's disease-linked variants of amyloid- β [31]. The predicted aggregation propensity is the strongest for sequence segments enriched in small hydrophobic and aromatic residues, but also Thr, Cys, Ser, Asn, and Gln. Sequences enriched in charged residues as well as Pro have the lowest tendency to aggregate [32]. A frequent occurrence of aggregation-prone hydrophobicity-enriched sequences is the price for

maintaining stability of globular proteins that rely on folding around hydrophobic cores. Interestingly, the TANGO analysis of multiple proteomes revealed that the aggregation-promoting sequences are preferentially capped with Arg or Lys [33]. This unexpected result suggested an electrostatic mechanism of counteracting protein aggregation that developed during a natural selection of protein sequences. Importantly, many molecular chaperones, including Hsp70 (DnaK) and Hsp100 (ClpB) show a preference towards predominantly basic sequences, in addition to the hydrophobic ones [22, 35]. The affinity of ClpB towards positively charged residues is also consistent with the known capability of poly-Lys to activate the ClpB ATPase [36]. Thus, it could be postulated that the chaperones evolved to recognize aggregation-prone motifs, including their positively charged “gatekeepers”. To our knowledge, the present study is the first attempt to test the above hypothesis for an Hsp100 chaperone.

The peptides B1 and B2 show a number of hallmark features attributed to the *bona fide* ClpB substrates: their interaction with ClpB requires ATP (see Fig. 3A), they stimulate the ClpB ATPase, compete with aggregated proteins for binding to ClpB, and shift the self-association equilibrium of ClpB towards the hexamer [22]. B1 and B2 are enriched in basic residues that are essential for recognition by ClpB (see Table I). Indeed, a replacement of the basic residues in B1 or B2 with alanines or with acidic residues abolished their binding to ClpB [22]. The TANGO analysis of B1 and B2 revealed that these peptides contain discrete aggregation-prone segments flanked by basic residues, consistent with the “gatekeeper” model (see Fig. 1).

Fluorescence-anisotropy titrations allowed us to obtain binding isotherms for the peptides interacting with ClpB in the ATP-bound state. We found that B1 and B2 become saturated with a hexameric ClpB at ~1:1 ratio (see Fig. 3B), which is consistent with the single substrate binding site located at the entrance to the ClpB channel [11, 22]. Interestingly, the peptide-ClpB interaction showed a positive cooperativity (see Fig. 3A). Since the cooperativity in binding of B1 and B2 to ClpB is not apparently associated with multiple binding sites (see Fig. 3B), it is likely a manifestation of an allosteric linkage between the ClpB-peptide interaction and the ClpB oligomerization, which was observed before [22, 37]. The nanomolar dissociation constants for the B1-ClpB and B2-ClpB complexes are consistent with the previous results [37].

We scrambled the sequences of B1 and B2 to modify their apparent aggregation-promoting properties. Interestingly, a swap between Phe within the aggregation-prone segment of B1 and Ala (see Table I) was sufficient to reduce the predicted aggregation index for the peptide B1N. Similarly, a redistribution of residues within B2 produced either a decrease or an increase of the predicted aggregation index in B2N, B2A, and B2B (see Fig. 1). We discovered that the peptides with the highest predicted aggregation index, i.e. B2A and B2 visibly aggregated in solution, as compared with the less aggregation-prone peptides (see Fig. 2), which validated the computational predictions.

Since ClpB is targeted towards aggregated substrates, rather than soluble misfolded proteins that are recognized by DnaK and GroEL [38], we hypothesized that the aggregation-prone sequences mediate interactions with ClpB. Unexpectedly, the peptides B1T, B2T1, and

B2T2, which contain only the predicted aggregating sequences (see Table I and Fig. 1) bind to ClpB with much lower affinities than the full-length B1 and B2 (see Fig. 4 and Table II). This result indicates that discrete aggregation-prone sequences, including their flanking basic residues are not sufficient for efficient interactions with ClpB. Moreover, we found that the ClpB binding affinities of the scrambled sequences B1N, B2N, B2A, and B2B were similar to those of the parent peptides B1 and B2 (see Fig. 5 and Table II). Thus, the aggregation-prone segments in the peptides are not necessary for efficient binding to ClpB.

We did not find a direct correlation between the overall aggregation propensity of the investigated peptides and their affinity towards ClpB. In spite of containing the aggregation-prone segments, the shorter peptides B1T, B2T1, and B2T2 showed a weak interaction with ClpB, which suggests that the length of the recognized peptide contributes to the binding to the chaperone, in agreement with a previously published result [37]. Quite unexpectedly, the peptides with a wide variability of the aggregation-propensity profiles (see Fig. 1) interacted with ClpB with similar affinities (see Fig. 5 and Table II). Notably, B1N interacted with ClpB with almost an order of magnitude lower K_d than B1, in spite of its insignificant aggregation score.

Our results suggest that the substrate recognition mechanism of ClpB may rely on multiple modes of interaction with broadly distributed motifs on the surface of aggregated particles, rather than on binding discrete local sequence segments. Possibly, the model peptides used in this study do not engage all substrate recognition sites in ClpB, such as those located in the N-terminal domain [23, 39], which limits their utility as mechanistic probes.

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Abbreviations:

FITC	fluorescein isothiocyanate
DMSO	dimethyl sulfoxide

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Highlights

- The mechanism of substrate recognition by the disaggregase ClpB is poorly understood.
- We investigated interactions of the substrate-mimicking peptides with ClpB.
- The aggregation-prone segments in peptides are not necessary for binding to ClpB.
- ClpB substrate recognition does not rely on local sequence motifs.

Figure 1. Prediction of the aggregation propensity of the peptides B1 (A), B2 (B), B2A (C), and B2B (D).

The aggregation index computed using the TANGO algorithm [31] is shown for the amino acid sequence of each peptide.

Figure 2. Aggregation propensity of the peptides in solution.

The solution turbidity was monitored at 40 °C after diluting the peptides to 150 μ M (see Materials and Methods).

Figure 3. ATP-dependent interaction between ClpB and the peptides B1 and B2.

The FITC fluorescence anisotropy changes were determined at room temperature upon titrating ClpB into the FITC-labeled peptide solutions. (A) Fluorescence anisotropy was determined for 18 nM peptides in 50 mM Tris-HCl, pH 7.5, 200 mM KCl, 20 mM MgCl₂ with 2 mM ATP or ADP. (B) Fluorescence anisotropy was determined for 80 nM peptides in 50 mM Tris-HCl, pH 7.5, 200 mM KCl, 20 mM MgCl₂ with 2 mM ATP.

Figure 4. Interaction between ClpB and the predicted aggregation-prone segments of the peptides B1 and B2.

The FITC fluorescence anisotropy changes were determined at room temperature upon titrating ClpB into the 18 nM peptide solutions in 50 mM Tris-HCl, pH 7.5, 200 mM KCl, 20 mM MgCl₂ with 2 mM ATP.

Figure 5. Interaction between ClpB and the peptides with different predicted aggregation propensities.

The FITC fluorescence anisotropy changes were determined at room temperature upon titrating ClpB into the 18 nM peptide solutions in 50 mM Tris-HCl, pH 7.5, 200 mM KCl, 20 mM MgCl₂ with 2 mM ATP.

Table I

Peptides used in this study

Peptide	Sequence
B1	AHAWQHQGKTLFISRKTYRI
B1T	KTLFISR
B1N	FHAWQHQGKTLAISRKTYRI
B2	QRKLFFNLRKTKQRLGWFNQ
B2T1	KLFFNLR
B2T2	RLGWFNQ
B2N	QLWQGQFRNLRRKTFFKKLN
B2A	QGQFRNLRRKQLWTFFLNKK
B2B	QQFRNRRKLTFLKKLGWFNQ

Table II

ClpB-peptide interaction parameters in the presence of ATP at 25 °C: dissociation constant (K_d) and Hill coefficient (h)

Peptide	K_d [nM]	h
B1	46.8 ± 1.5	2.40 ± 0.18
B1T	329 ± 39	1.72 ± 0.14
B1N	7.26 ± 0.32	1.64 ± 0.13
B2	13.24 ± 0.42	2.52 ± 0.22
B2T1	912 ± 231	1.13 ± 0.06
B2T2	>1,000	not determined
B2N	17.54 ± 0.92	1.85 ± 0.19
B2A	10.17 ± 0.80	1.31 ± 0.17
B2B	11.51 ± 0.52	1.47 ± 0.11