Poly-L-lysine enhances the protein disaggregation activity of ClpB

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Abstract The Hsp100 protein ClpB is a member of the AAA+ protein family that mediates the solubilization of aggregated proteins in cooperation with the DnaK chaperone system. Unstructured polypeptides such as casein or poly-L-lysine have been shown to stimulate the ATPase activity of ClpB and thus may both act as substrates. Here we compared the effects of α-casein and poly-L-lysine on the ATPase and chaperone activities of ClpB. α-Casein stimulated ATP hydrolysis by both AAA domains of ClpB and inhibited the ClpB-dependent solubilization of aggregated proteins if present in excess. In contrast, poly-L-lysine stimulated exclusively the ATPase activity of the second AAA domain and increased the disaggregation activity of ClpB. Thus poly-L-lysine does not act as substrate, but rather represents an effector molecule, which enhances the chaperone activity of ClpB.

Key words: Protein disaggregation; Chaperone; ClpB; DnaK; AAA+ protein

1. Introduction

The chaperone ClpB of *Escherichia coli* belongs to the ring-forming AAA+ protein family, which comprises AAA (ATPase associated with a variety of cellular activities) and Clp/Hsp100 proteins [1]. Both protein classes share considerable sequence homology in regions which are important for ATP hydrolysis and oligomerization, termed AAA domains. Each AAA domain consists of a core ATPase domain, containing the classical Walker A and B motifs, and a C-terminal α-helical domain (referred to as C domain). Generally AAA+ proteins drive the assembly and disassembly of protein complexes by ATP-dependent remodelling of protein substrates [2,3]. ClpB mediates the solubilization of aggregated proteins in cooperation with the DnaK chaperone system [4–7]. ClpB consists of two AAA domains, separated by a 'linker' region and an N-terminal domain [8]. Both AAA domains are essential for ClpB oligomerization and activity [9–12]. The functions of the N domain and the 'linker' segment are currently unknown. While N domains are dispensable for the disaggregating activity of ClpB, the 'linker' region has an essential function in this process [12,13].

Substrate specificity of ClpB and the localization of substrate binding sites are unknown. Unstructured polypeptides like casein and poly-L-lysine can stimulate the ATPase activity of ClpB and thus may act as ClpB substrates [13–16]. It was recently suggested that lysine residues represent the primary recognition motifs in protein substrates, since poly-L-lysine inhibited the chaperone activity of Hsp104, the ClpB homologue of *Saccharomyces cerevisiae* [16]. Interestingly, poly-L-lysine interacted with the C domain of the second AAA domain of Hsp104 [16]. Isolated C domains of AAA+ chaperones have been demonstrated to interact with substrates and were therefore termed the sensor and substrate discrimination (SSD) domain [17]. However, the contribution of the SSD domain to substrate binding in the context of the full-length proteins is still unclear.

Clp/Hsp100 proteins were originally identified by their ability to degrade casein [18–20]. However, there are conflicting data on whether casein can be used as model substrate for ClpB. On the one hand, mutations of conserved residues in the N domain of *E. coli* result in both loss of activity in protein disaggregation and failure of casein to stimulate the ATPase activity of ClpB, suggesting substrate-like features of casein. [15]. On the other hand, deletion of the entire N domain did not affect chaperone activity of ClpB of *E. coli* and *Thermus thermophilus*, but caused a reduced ATPase stimulation by casein [12,13]. Reinstein and co-workers demonstrated that the reduced stimulation of the *T. thermophilus* ΔN-ClpB ATPase activity by casein is caused by a reduced binding affinity for this protein [13]. Interestingly, ClpB variants with alterations in the N domain, while exhibiting reduced ATPase stimulation by casein, were still fully stimulatable by poly-L-lysine [13,15].

In order to clarify the relevance of the interaction of ClpB with either polypeptide, we compared the influence of poly-L-lysine and α-casein on the ATPase and chaperone activity of ClpB. We demonstrate that poly-L-lysine does not act as substrate of ClpB, but rather represents an effector molecule which enhances ClpB activity by accelerating ATP hydrolysis by the second AAA domain. In contrast, α-casein enhanced the ATPase activity of both AAA domains and inhibited protein disaggregation by ClpB/KJE if present in excess. Since α-casein also inhibited the chaperone activity of a ClpB deletion variant missing the N domain, multiple casein binding sites must exist within ClpB.

2. Materials and methods

2.1. Proteins and polypeptides

Purifications of DnaK, DnaJ, GrpE and ClpB were performed as described previously [6,21]. Pyruvate kinase, α-casein, poly-L-lysine

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and poly-L-arginine were purchased from Sigma, malate dehydrogenase (MDH) from pig heart muscle and firefly luciferase were from Roche. Protein concentrations were determined with the Bio-Rad Bradford assay using bovine serum albumin as standard. Concentrations of poly-L-lysine and poly-L-arginine were calculated based on the average molecular weight given by the manufacturer. Protein concentrations refer to the protomer.

2.2. ATPase activity assay

ATP hydrolysis rates under steady-state conditions were determined as described [21]. Reactions were performed at 30°C in buffer A (50 mM Tris, pH 7.5; 150 mM KCl; 20 mM $MgCl_2$; 2 mM dithiothreitol) containing 0.5 μM ClpB (wild type or derivatives), 2 mM ATP and [α-^{32}P]ATP (0.1 μCi, Amersham). Hydrolysis was quantified using the program MACBAS version 2.5 (Fuji) and rates of ATP hydrolysis were determined using the program GRAFIT version 3.0 (Erithacus software).

2.3. Protein denaturation and chaperone activity assays

MDH (2 μM) was denatured at 47°C for 30 min in buffer A. Firefly luciferase (0.2 μM) was denatured in buffer A at 43°C for 15 min. Protein refolding was started by incubating aggregated proteins and chaperones in buffer A at 30°C. All assays were performed in the presence of an ATP-regenerating system (3 mM phosphoenol pyruvate; 20 μg/ml pyruvate kinase; 2 mM ATP). Determination of enzymatic activities followed published protocols [4,22]. For light scattering measurements MDH or luciferase were denatured as described above. Turbidity was measured at an excitation and emission wavelength of 550 nm (Perkin-Elmer luminescence spectrometer LS50B). Decrease of turbidity was followed upon addition of indicated chaperones in the presence of an ATP-regenerating system at 30°C. The amount of soluble [^{3}H]MDH species at different time points of the disaggregation reaction was determined by scintillation counting of soluble and insoluble fractions after sample centrifugation (13 000 rpm for 30 min at 4°C). Labeling of MDH was performed by use of *N*-succinimidyl[2,3-^{3}H]propionate (Amersham) as described [23].

3. Results

3.1. Poly-L-lysine stimulates ATP hydrolysis by ClpB in a length-dependent manner

We performed a dose response of the *E. coli* ClpB ATPase activity in the presence of increasing poly-L-lysine and α-casein concentrations. In the case of poly-L-lysine three variants of different sizes (1–4 kDa, 4–15 kDa and 15–30 kDa) were tested since a strong influence of poly-L-lysine chain length on the ATPase activity of Hsp104 has been reported [16]. As for Hsp104 [16], poly-L-lysine stimulated the ATPase activity of ClpB in a chain length-dependent manner. While a shorter version of poly-L-lysine (1–4 kDa) did not exhibit stimulatory effects, longer derivatives (4–15 kDa and 15–30 kDa) increasingly enhanced the ATPase activity of ClpB up to 25-fold (Fig. 1). Half-maximal stimulation was determined in the presence of 20 μM poly-L-lysine (4–15 kDa) and 0.5 μM poly-L-lysine (15–30 kDa). Interestingly, poly-L-arginine (5–15 kDa) did not stimulate ClpB ATPase activity, indicating that stimulation may not simply be caused by abundant positive charges. Saturating concentrations of α-casein induced the ATPase activity of ClpB wild type five-fold and thus quantitatively differed significantly from the ATPase stimulation by poly-L-lysine.

3.2. Poly-L-lysine, in contrast to α-casein, exclusively increases the ATPase activity of the second AAA domain

We analyzed the stimulation pattern of both potential ClpB substrates by determining their influence on the ATPase activity of a variety of ClpB mutants, including deletion variants missing the N domain or the 'linker' region, and Walker B

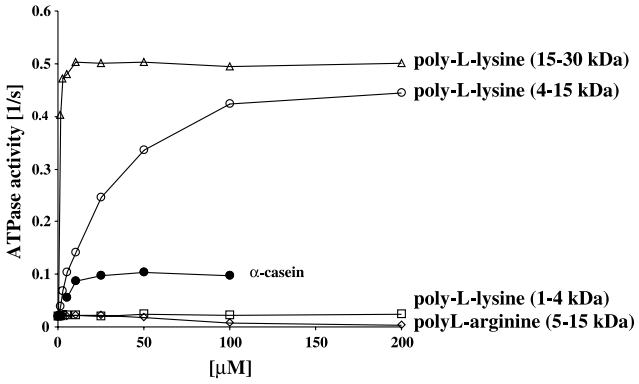

Fig. 1. Poly-L-lysine and α-casein increase the ATPase activity of ClpB. Specific ATPase activities of ClpB (0.5 μM) were determined in the presence of increasing poly-L-lysine, poly-L-arginine and α-casein concentrations.

mutants, defective in ATP hydrolysis in the first or second AAA domain (Fig. 2). None of the mutants exhibits oligomerization defects [12]. α-Casein (10 μM) induced the ATPase activity of ClpB variants missing the N domain or the 'linker' region by two-fold or eight-fold, respectively. Poly-L-lysine stimulated the ATPase activity of the deletion variants much more strongly (approx. 20-fold), comparable to ClpB wild type. Analysis of ClpB Walker B mutants (E279A, E678A), which eliminate the ATPase activity of the corresponding AAA domain without affecting ATP binding, revealed that α-casein stimulated ATP hydrolysis by both AAA domains to similar degrees. In contrast, poly-L-lysine only activated the second AAA domain, since it only slightly stimulated ATP hydrolysis by ClpB E678A (Fig. 2). Consistently, α-casein but not poly-L-lysine accelerated ATP hydrolysis by ClpB 813AAA815. This mutant of the highly conserved 813GAR815 motif in the C domain of the second AAA module exhibits defects in ATP binding to the second AAA domain [12]. We conclude that poly-L-lysine, in contrast to α-casein, exclusively stimulates ATP hydrolysis by the second AAA domain of ClpB.

3.3. Poly-L-lysine enhances the disaggregation activity of ClpB

To investigate whether α-casein and poly-L-lysine act as substrates for ClpB we tested their ability to interfere with the chaperone activity of ClpB. For this purpose we followed

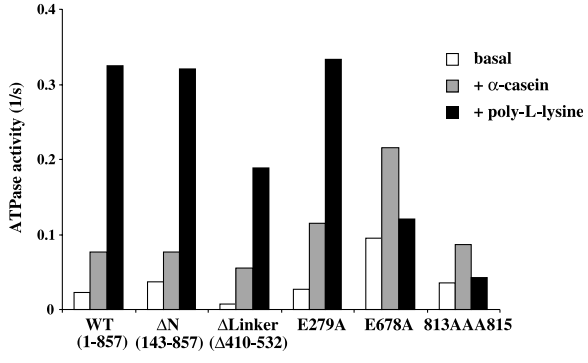

Fig. 2. Poly-L-lysine exclusively stimulates ATP hydrolysis by the second AAA domain of ClpB. Specific ATPase activities of ClpB deletion variants and mutants were determined in the absence or presence of 50 μM poly-L-lysine (4–15 kDa) or 10 μM α-casein.

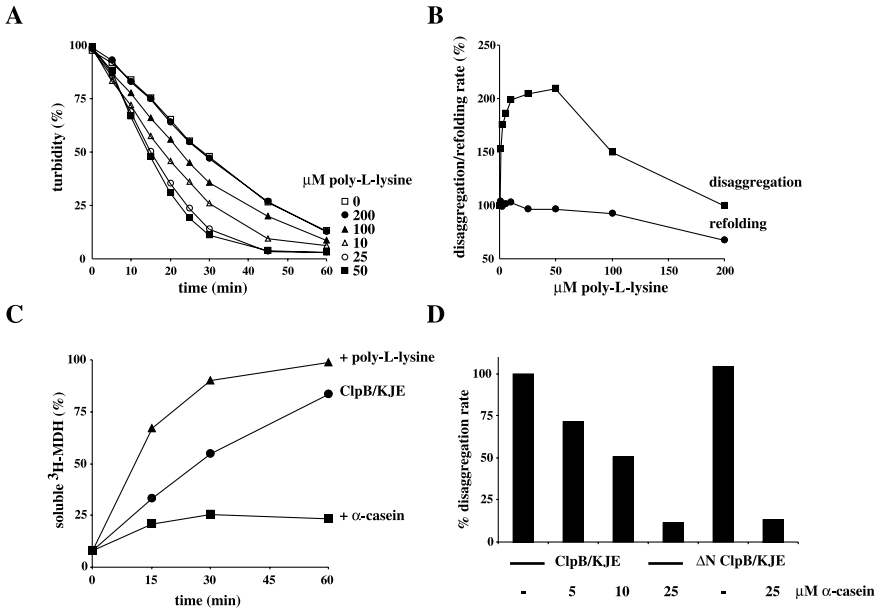

Fig. 3. Poly-L-lysine increases the chaperone activity of ClpB. A: Disaggregation of 1 μM heat-aggregated MDH was initiated at 30°C by addition of the DnaK system (1 μM DnaK, 0.2 μM DnaJ, 0.1 μM GrpE) and 0.5 μM ClpB in the absence or presence of increasing poly-L-lysine (4–15 kDa) concentrations as indicated. B: MDH disaggregation and refolding rates were determined based on the linear decrease of aggregate turbidity or the linear increase in MDH activity, respectively. Disaggregation and refolding rates calculated in the absence of poly-L-lysine were set as 100%. C: MDH disaggregation by ClpB/KJE (squares) was followed by determining the amount of soluble [^{3}H]MDH species in the additional presence of 50 μM poly-L-lysine (4–15 kDa) (triangles) or 25 μM α-casein (squares). D: MDH disaggregation rates were determined based on the linear increase in [^{3}H]MDH solubility in the presence of ClpB/KJE (concentrations as in A) and increasing α-casein concentrations. Disaggregation rates determined in the absence of α-casein were set as 100%.

the solubilization of heat-aggregated MDH by ClpB/KJE in the additional presence of either polypeptide (Fig. 3). Solubilization of MDH aggregates was directly followed by measuring the decrease in aggregate turbidity upon ClpB/KJE addition. MDH aggregates were disaggregated faster in the presence of 10–100 μM poly-L-lysine (4–15 kDa) (Fig. 3A). Similar results were obtained when poly-L-lysine (15–30 kDa) was added to the disaggregation reaction (data not shown). This acceleration of the disaggregation reaction could be attributed to enhanced chaperone activity of ClpB, since poly-L-lysine had no effects on the refolding rates of solubilized MDH molecules (Fig. 3B), which primarily reflect DnaK function [4]. MDH disaggregation rates were increased by up to two-fold and fastest solubilization occurred when 50 μM poly-L-lysine was added to the bi-chaperone system. Half-maximal stimulation of MDH disaggregation was already obtained in the presence of 1 μM poly-L-lysine, indicating that a two-fold stimulation of the ATPase activity is already sufficient to enhance ClpB activity. Higher poly-L-lysine concentrations continuously reduced the stimulatory effect, probably by affecting the subsequent refolding of solubilized MDH molecules (Fig. 3B). The stimulatory effect of poly-L-lysine was also observed in order-of-addition experiments, when poly-L-lysine was added after 10 min to an ongoing disaggregation reaction (data not shown).

Addition of 50 μM poly-L-lysine (4–15 kDa) to either ClpB (1.5 μM) or the DnaK system (1 μM DnaK) alone did not result in any MDH disaggregation, confirming that only the cooperative action of ClpB and KJE allows solubilization of large aggregates (data not shown). Solubilization of MDH aggregates by ClpB/DnaK was not stimulated at any tested concentration of a shorter poly-L-lysine version (1–4 kDa), indicating that acceleration of ATP hydrolysis by poly-L-lysine (4–15 kDa or 15–30 kDa) and the stimulation of ClpB chaperone activity are coupled (data not shown).

Light scattering measurements in the presence of α-casein were obstructed by a strong increase in sample turbidity upon addition of α-casein. We therefore additionally followed the solubilization of MDH aggregates by determining the amount of soluble MDH species generated during the disaggregation reaction (Fig. 3C). In order to allow a more precise quantification, MDH was labelled with ^{3}H. Labelled MDH was fully active and exhibited the same aggregation and disaggregation kinetics as non-labelled MDH (data not shown). In the presence of ClpB/KJE soluble [^{3}H]MDH species were recovered from the aggregates with the same kinetics as the turbidity of MDH aggregates decreased. In agreement with the determined faster decrease of MDH turbidity, soluble [^{3}H]MDH species occurred more quickly during the disaggregation reaction when 25 μM poly-L-lysine (4–15 kDa) was added to the bi-chaperone system (Fig. 3C). In contrast, presence of 25 μM α-casein strongly inhibited MDH solubilization and, consistently, an inhibition of MDH refolding was noticed at the same time (data not shown). Titration of α-casein revealed that an excess of casein is necessary to block MDH disaggregation. The inhibitory effect of α-casein was not caused by its interaction with the N domains of ClpB, since the activity of ΔN ClpB was affected to the same degree (Fig. 3D).

Finally we determined the consequences of varying ClpB/KJE levels on the stimulatory effect of poly-L-lysine on the disaggregation reaction (Fig. 4). Either component of the bi-chaperone system was titrated while keeping the concentration of the partner chaperone constant. Strongest stimulation (5.5-fold) of the disaggregation reaction by poly-L-lysine was observed when ClpB (0.1 μM) was limiting and stimulation factors became smaller with increasing ClpB concentrations. In

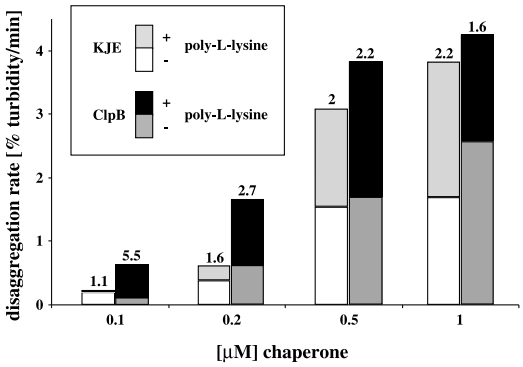

Fig. 4. Effects of poly-L-lysine on the disaggregation reaction in the presence of varying ClpB/KJE ratios. ClpB or KJE levels were varied in the absence or presence of 25 μM poly-L-lysine (4–15 kDa). In the case of ClpB titration KJE levels were kept constant (1 μM DnaK, 0.2 μM DnaJ, 0.1 μM GrpE) and ClpB (0.5 μM) stayed constant when KJE was titrated. The ratio of K:J:E was kept constant throughout the titration. The given chaperone concentrations refer to either ClpB or DnaK. MDH disaggregation rates were determined in the absence or presence of poly-L-lysine. The stimulation factors of the disaggregation reaction in presence of poly-L-lysine are given.

contrast, low KJE concentrations could not be overcome by addition of poly-L-lysine while increasing KJE levels restored the stimulatory effect (Fig. 4). These data confirm the exclusive stimulatory effect of poly-L-lysine on ClpB and the interdependence of both chaperone systems for efficient protein disaggregation: activation of ClpB only leads to enhanced protein disaggregation when sufficient KJE levels are provided.

3.4. Poly-L-lysine stimulates the disaggregation of aggregated luciferase but inhibits luciferase refolding

While poly-L-lysine accelerated the solubilization of MDH aggregates by ClpB/KJE, it has been shown to inhibit the refolding of aggregated luciferase by Hsp104/Hsp70 from yeast [16]. We therefore also investigated the consequences of poly-L-lysine on the solubilization of heat-aggregated luciferase by ClpB/KJE (Fig. 5). In agreement with published data for the Hsp104/Hsp70 bi-chaperone system, the refolding of luciferase by ClpB/KJE was continuously inhibited in the presence of increasing poly-L-lysine (4–15 kDa) concentrations. However, a different result was obtained when solubilization of luciferase aggregates was directly followed by measuring the decrease in aggregate turbidity: Low poly-L-lysine concentrations (up to 10 μM) stimulated luciferase disaggregation, while high concentrations (above 25 μM) slightly inhibited disaggregation (Fig. 5). The most plausible explanation of these findings is that poly-L-lysine rather blocks the refolding of solubilized luciferase but does not inhibit ClpB activity. To test this possibility directly we analyzed the effects of poly-L-lysine on the refolding of non-aggregated, heat-denatured luciferase. Luciferase aggregation was prevented by the addition of KJ during heat denaturation at 43°C. Luciferase refolding was initiated at 30°C by addition of GrpE in the presence of increasing poly-L-lysine concentrations (Fig. 5). Refolding of luciferase by KJE was inhibited even more strongly by poly-L-lysine, confirming that the reported inhibitory effects of poly-L-lysine are not caused by affecting ClpB activity. Notably, increasing concentrations of KJE could restore luciferase refolding in presence of poly-L-lysine (data not shown).

3.5. Stimulation of the second ATPase domain by poly-L-lysine is essential for enhancing ClpB chaperone activity

We next asked whether the stimulation of the second AAA domain is essential for the beneficial effects of poly-L-lysine on the chaperone activity of ClpB. To answer this question we chose ClpB variants with mutational alterations in the Walker B motifs (E279A, E678A), which are deficient in ATP hydrolysis in the corresponding AAA domain, but still retain some partial activity in disaggregating and refolding aggregated MDH [12]. MDH disaggregation by a Walker B mutant of the first ATPase domain (E279A) was activated 1.8-fold by poly-L-lysine (4–15 kDa), similar to ClpB wild type or a ClpB variant missing the N domain (Fig. 6). The increased chaperone activity of ClpB E279A was still low (7% of ClpB wild type control), confirming that two functional AAA domains are needed for efficient protein disaggregation. The partial chaperone activity of the second Walker B mutant (E678A), whose ATPase activity is not stimulated by poly-L-lysine, was not increased. Thus stimulation of the second ATPase domain by poly-L-lysine is directly coupled to the activation of ClpB.

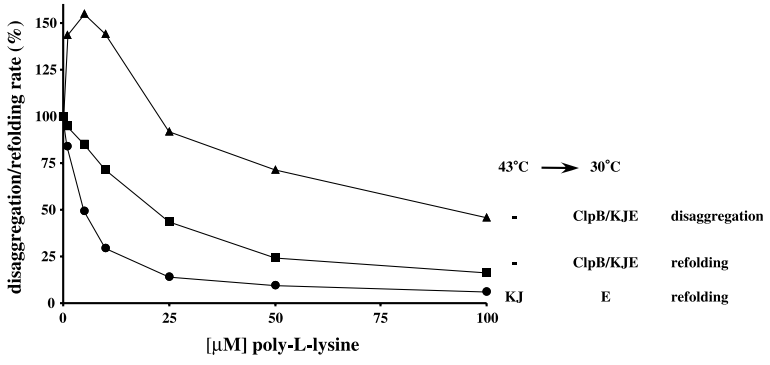

Fig. 5. Poly-L-lysine accelerates solubilization of luciferase aggregates by ClpB/KJE but inhibits the subsequent refolding reaction. 0.1 μM luciferase was denatured at 43°C in the absence or presence of 1 μM DnaK and 0.2 μM DnaJ. Disaggregation and refolding of aggregated luciferase was initiated at 30°C by addition of the DnaK system (1 μM DnaK, 0.2 μM DnaJ, 0.1 μM GrpE) and 1 μM ClpB in the absence or presence of increasing poly-L-lysine (4–15 kDa) concentrations. Refolding of soluble, unfolded luciferase, generated by denaturation in the presence of 1 μM DnaK, 0.2 μM DnaJ and 2 mM ATP, was started by addition of 0.1 μM GrpE. Luciferase disaggregation and refolding rates were determined based on the linear decrease of aggregate turbidity or the linear increase of luciferase activity, respectively. Disaggregation and refolding rates calculated in the absence of poly-L-lysine were set as 100%.

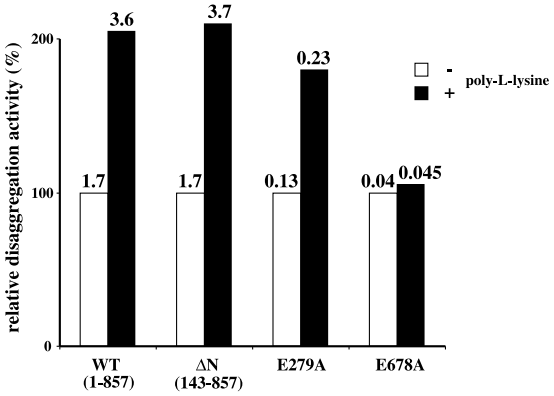

Fig. 6. Stimulation of ClpB ATPase activity by poly-L-lysine is linked to an increased disaggregation activity. Resolubilization of 1 μM aggregated MDH by the DnaK system (1 μM DnaK, 0.2 μM DnaJ, 0.1 μM GrpE) and 0.5 μM ClpB wild type or ClpB ΔN was followed in the absence or presence of 50 μM poly-L-lysine (4–15 kDa). In the case of ClpB Walker B mutants (E279A, E678A) 1.5 μM was used due to their low chaperone activity. MDH disaggregation activities determined in the absence of poly-L-lysine were set as 100% for each ClpB variant. Absolute MDH disaggregation rates (% turbidity/min) of each ClpB variant are given.

4. Discussion

In this study we investigated the effects of α-casein and poly-L-lysine on the ATPase and chaperone activities of the AAA+ protein ClpB. α-Casein and poly-L-lysine differed in their consequences on the ClpB ATPase stimulation profile, indicating that they interact with different sites within ClpB. Poly-L-lysine stimulated exclusively the second AAA domain of ClpB in a length-dependent manner. Very similar effects of poly-L-lysine on the ATPase activity of *S. cerevisiae* Hsp104 have been published recently [16], implying that poly-L-lysine interacts with both AAA+ proteins in the same manner. Interestingly, poly-L-lysine also stimulates the ATPase activity of the AAA+ protein HslU [24]. HslU consists of only one AAA domain, which is homologous to the second AAA domain of ClpB and Hsp104. The effects of poly-L-lysine on the ATPase activity of this AAA domain might therefore be conserved among Hsp100/Clp proteins.

The mechanism by which poly-L-lysine accelerates ATP hydrolysis by the second AAA domain of ClpB is unknown. Stimulation was independent of a functional first ATPase domain and the presence of the N domain and the 'linker' region, suggesting that the interaction of poly-L-lysine with ClpB is restricted to the second AAA domain. Indeed, binding of poly-L-lysine to isolated C domains of the second AAA domain has been demonstrated for Hsp104 [16]. Binding of poly-L-lysine to the C domains may coordinate ATP hydrolysis by interacting with multiple ClpB subunits, as suggested by the finding that stimulation of the ATPase activity was dependent on chain length. Importantly, the arginine residue of the invariant GAR motif within the C domain contacts the γ phosphate of bound ATP and may stimulate ATP hydrolysis upon binding of poly-L-lysine.

While the effects of poly-L-lysine on the ATPase activities of ClpB and Hsp104 are very similar, the consequences on the chaperone activity are fundamentally different. Poly-L-lysine has been proposed to act as substrate for *S. cerevisiae* Hsp104 and, consistent with this suggestion, it strongly inhibited refolding of aggregated luciferase by the Hsp104/Hsp70 bi-chaperone system [16]. In contrast, poly-L-lysine increased the disaggregating activity of *E. coli* ClpB, instead of inhibiting it, as would be expected if poly-L-lysine acted as a competing substrate. Stimulation of ClpB activity by poly-L-lysine was shown for heat-aggregated MDH and luciferase. Interestingly, poly-L-lysine blocked luciferase refolding from aggregates by ClpB/KJE, consistent with published findings for Hsp104/Hsp70. However, this inhibitory effect was restricted to the refolding of solubilized, unfolded luciferase and was not caused by reduced ClpB activity. Since KJE-mediated refolding of the alternative substrate MDH was not strongly influenced by poly-L-lysine, we suggest that poly-L-lysine affects the refolding of non-native luciferase molecules per se, independent of KJE.

Taken together we propose that poly-L-lysine represents an effector molecule, rather than a ClpB substrate, that enhances the chaperone activity of ClpB by accelerating ATP hydrolysis in the second AAA domain. Importantly, poly-L-lysine also stimulated the proteolytic activity of the HslU/HslV protease, suggesting that the effects of poly-L-lysine on the chaperone activity of Hsp100/Clp proteins are conserved [24].

In contrast to poly-L-lysine, α-casein inhibited the disaggregation activity of ClpB. Notably, a strong decrease in MDH disaggregation rates was only observed in the presence of an excess of α-casein, at conditions at which the casein-stimulatable ATPase activity was already saturated. This observation can be potentially explained by the heterogeneous nature of casein, which is not a defined species. Thus casein species which stimulate the ATPase activity of ClpB by binding to the N domains may be different from those which inhibit the ClpB chaperone activity by interacting with different binding sites within ClpB. Consistently, ClpB inhibition was not caused by interaction of α-casein with N domains since ΔN ClpB was also affected in its chaperone activity upon α-casein addition. Furthermore, the ATPase activity of ΔN ClpB was still stimulatable by α-casein. We therefore propose that additional casein binding sites must exist within ClpB, potentially in the first AAA domain. Consistent with this suggestion, ΔN ClpA is still able to prepare casein for degradation by ClpP, albeit with a lower efficiency compared to full-length ClpA [25]. Finally, ATP-dependent binding of α-casein to a ClpB variant with mutational alterations in the Walker B motif of both AAA domains (E279A/E678A), which binds but does not hydrolyze ATP, could be demonstrated recently. Importantly, a mutant variant missing the N domains was not affected in casein interaction, confirming the existence of additional casein binding sites within ClpB [26].

In summary, we have demonstrated that poly-L-lysine represents an effector molecule which enhances the disaggregation activity of ClpB. Interestingly, Goloubinoff and co-workers could demonstrate that K-glutamate and glycine-betaine, components which are accumulated in salt-stressed *E. coli* cells, also stimulate protein disaggregation by ClpB/KJE [27,28]. In vitro glycine-betaine could preserve the oligomeric state of ClpB under high salt conditions, indicating an additional function of this osmolyte during salt stress in vivo. Thus effector molecules could add a new level to regulate the activity of ClpB and/or other Hsp100/Clp proteins and may allow chaperone activation in response to specific environmental changes.

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