The AAA+ protein ClpB mediates the solubilization of protein aggregates in cooperation with the DnaK chaperone system (KJE). The order of action of ClpB and KJE on aggregated proteins is unknown. We describe a ClpB variant with mutational alterations in the Walker B motif of both AAA domains (E279A/E678A), which binds but does not hydrolyze ATP. This variant associates in vitro and in vivo in a stable manner with protein substrates, demonstrating direct interaction of ClpB with protein aggregates for the first time. Substrate interaction is strictly dependent on ATP binding to both AAA domains of ClpB. The unique substrate binding properties of the double Walker B variant allowed to dissect the order of ClpB and DnaK action during disaggregation reactions. ClpB-E279A/E678A outcompetes the DnaK system for binding to the model substrate TrfA and inhibits the dissociation of small protein aggregates by DnaK only, indicating that ClpB acts prior to DnaK on protein substrates.

Numerous cellular activities rely upon ATP binding and hydrolysis by AAA+ (ATPase-associated with various cellular activities) proteins (1). AAA+ proteins self-assemble into oligomeric structures and convert the energy derived from ATP hydrolysis into mechanical force to remodel their target substrates (2, 3). In *Escherichia coli* many AAA+ proteins are members of the Hsp100 chaperone family (ClpA, ClpB, ClpX, HslU). Hsp100 proteins associate with a peptidase to form proteolytic machines, which degrade damaged and naturally unstable proteins. The Hsp100 constituents of these machines are responsible for recognition, unfolding, and translocation of the substrates into the proteolytic chamber of the associated peptidase (4–6). ClpB is unique among the Hsp100 proteins of *E. coli* since it does not interact with a proteolytic partner protein. Instead ClpB cooperates with the DnaK chaperone system (DnaK, DnaJ, GrpE; KJE) in the solubilization and refolding of aggregated proteins (7–11). The disaggregating activity of ClpB is essential for the development of thermotolerance in *E. coli*, but also in *Saccharomyces cerevisiae* and *Arabidopsis thaliana* (12–14).

ClpB consists of a N-terminal domain and two AAA domains, which are separated by a “linker” region (15). AAA domains mediate ATP binding and hydrolysis and are essential for ClpB oligomerization (16–19). 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 (16, 20).

The mechanism of the disaggregation reaction and the functional interplay between ClpB and KJE is not understood. KJE is able to dissociate small protein aggregates and protein complexes in the absence of ClpB and associate unproductively with large protein aggregates (8, 10, 21, 22). ClpB strongly increases the efficiency of KJE in dissociating small aggregates and expands the substrate spectrum of the DnaK system by enabling KJE to dissociate large aggregates (7, 21). Surprisingly, ClpB has no detectable chaperone activity on its own and does not even dissociate small protein complexes (7, 9, 21, 23). A major problem to dissect the disaggregation reaction mechanistically is the highly transient nature of ClpB substrate interaction. Direct interactions between ClpB and protein aggregates have so far not been demonstrated. Thus aggregates isolated from heat-shocked *E. coli* cells do not retain ClpB and wild-type ClpB has not been detected in complex with aggregated substrates in vitro (10). Since DnaK is associated with heat-aggregated proteins, the order of ClpB and KJE action on protein aggregates remains unclear.

Stabilized substrate interactions have been achieved for some AAA+ proteins in the presence of non-hydrolyzable ATP analogs (24, 25). However, interaction of substrates with Hsp104, the ClpB homologue in *Saccharomyces cerevisiae*, is not stabilized by nucleotide analogs (26). To overcome this problem we searched in this study for ClpB variants exhibiting stable interaction with substrate proteins. This criterion was only fulfilled by a ClpB variant with mutational alterations in the Walker B motifs of both AAA domains (E279A/E678A), referred to as ClpB-B1/2A, which binds but does not hydrolyze ATP. Substrate interaction was strictly ATP-dependent and stabilization of substrate binding needed inactivation of ATP hydrolysis in both AAA domains. ClpB-B1/2A allowed to dissect the order of ClpB and DnaK action on aggregated proteins by analyzing the effects of ClpB-E279A/E678A on DnaK binding to protein complexes, which serve as substrates for DnaK and ClpB. ClpB-B1/2A out competed DnaK in binding to TrfA, a DNA-binding protein which regulates the replication of oriV-containing plasmids (23) and inhibited dissociation of small protein aggregates by KJE, indicating that ClpB is acting prior to DnaK during the disaggregation reaction.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—*E. coli* strains used were derivatives of MC4100 (araD139 DArgF-loclU169 rpsL150 relA1 rpsL150 relA1 deoC1 ppsF25 rbsR). The *E. coli* ndk gene was replaced by a kanamycin resistance cassette by usage of the oligonucleotides 5'-AGGCTGGAGCTGCTTCG and 3'-ndk, 5'-GCTGGTACAGACAACAACAGAACAATTTACAGAGGTAAAAGTGT-3'. The *E. coli ndk gene was replaced by a kanamycin resistance cassette by usage of the oligonucleotides 5'-AGGCTGGAGCTGCTTCG and 3'-ndk, 5'-GCTGGTACAGACAACAACAGAACAATTTACAGAGGTAAAAGTGT-3'.

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clpB (E279A/E678A) were generated by standard cloning techniques in pUHE21–26D12.

Proteins—Wild-type and mutant ClpB were purified as described after overproduction in ΔclpBΔrsk cells (16). Purifications of DnaK, DnaJ, GrpE, GroEL/D87K, Hsp16.6, ClpC, and MecA were according to published protocol (7, 10, 25, 29). DnaK was purified from ΔdnkBΔrsk mutant cells to avoid co-purification of nucleoside-diphosphate kinase (30). N-terminally His6-tagged TrfA was purified by Ni-NTA affinity chromatography following standard protocols given by the manufacturer (Qiagen). In vivo labeling of TrfA was performed in M9 minimal medium supplemented with 0.2% (w/v) glucose and all L-amino acids (50 mg/ml amino acid) except t-methionine. Labeling was achieved by adding 0.7 mCi of L-[35S]methionine (Amersham Biosciences SJ1515; 15 mCi/ml, 1000 Ci/mmol) during TrfA overproduction to the cell culture. Pyruvate kinase and α-casein were purified from Sigma, malate dehydrogenase (MDH) from pig heart muscle and firefly luciferase from Roche Applied Science. ClpB-B1/2A, α-casein, and MDH were labeled by use of N-succinimidyl [2,3-3H]propionate (Amersham Biosciences) as described (31). Protein concentrations were determined with the Bio-Rad Bradford assay using bovine serum albumin as standard.

ATPase Assays—DnaK-ATP complexes were prepared and tested for ATP hydrolysis in single-turnover assays as described (32), with a final concentration of DnaK of 0.5 μM. Rate constants were determined with the program GRAFIT (Erithacus Software, Staines, UK).

Prevention of MDH and Luciferase Aggregation—[3H]MDH (1 μM) was denatured at 47 °C for 30 min in buffer A (50 mM Tris, pH 7.5, 150 mM KCl, 20 mM MgCl2, 2 mM dithiothreitol) in the absence or presence of chaperones. Soluble and aggregated MDH species were separated by centrifugation (13000 rpm, 30 min, 4 °C) and the amount of each fraction was quantified by scintillation counting. Luciferase (0.1 μM) was denatured in buffer A with or without chaperones at 43 °C. Aggregation of luciferase was monitored as an increase in sample turbidity. Turbidity was measured at an excitation and emission wavelength of 550 nm (PerkinElmer luminescence spectrometer LS50B).

Refolding of MDH from sHsp/MDH Complexes—[3H]MDH (1 μM) was denatured at 47 °C for 30 min in buffer A in the presence of Hsp16.6 (4 μM). MDH was reconstituted by diluting Hsp16.6/MDH complexes and chaperones in buffer A at 30 °C. All assays were performed in the presence of an ATP-regenerating system (3 mM phosphoenolpyruvate; 20 μg/ml pyruvate kinase; 2 mM ATP). Determination of enzymatic activities followed published protocols (7). Refolding rates were calculated from the linear increase of substrate activities.

Isolation of Aggregated Proteins, SDS-PAGE, Immunoblotting, and Protein Quantification—Protein aggregates from E. coli cells were isolated as described (33). Gel electrophoresis was carried out according to Laemmli (34) using 15% SDS-polyacrylamide gels and staining with Coomassie Brilliant Blue. Immunoblotting was performed according to standard procedures, using rabbit antisera specific for IbpB (exhibiting secondary antibodies) or ClpB as primary antibody, and developed with a Vistra ECF fluorescence immunoblotting kit (Amersham Biosciences) using alkaline phosphatase-conjugated anti-rabbit IgG as secondary antibodies (Vector Laboratories). Developed immunoblots were scanned using a fluorimager (FLA-2000) and quantified using MacBAS software (Fuji Film).

RESULTS

ClpB-B1/2A Prevents the Aggregation of Thermolabile Model Substrates in an ATP-dependent Manner—For the mechanistic analysis of ClpB/KJE-dependent protein disaggregation we were interested to identify variants of ClpB, which interact with protein substrates in a stable manner. In a first approach, binding of ClpB to unfolded proteins was tested by a prevention of aggregation assay. The heat-induced aggregation of thermolabile model proteins (MDH, firefly luciferase) was monitored in the additional presence of the chaperone. MDH aggregation was followed by determining the amount of insoluble MDH species after heat denaturation. In order to allow a precise calculation of aggregated and soluble MDH species, MDH was labeled with 3H. Labeled MDH was fully active and exhibited the same aggregation kinetics as non-labeled MDH (data not shown). More than 90% of MDH molecules became insoluble after temperature-upshift to 47 °C and could be pelleted by centrifugation (Fig. 1A). Experiments were started with a ClpB mutant altered in the Walker B motif of both AAA domains (E279A/E678A, also referred to as B1/2A). ClpB-B1/2A forms oligomers, binds but does not hydrolyze ATP and exhibits increased resistance toward proteolysis compared with ClpB wild type (16). In the presence of 2 mM ATP ClpB-B1/2A prevented the aggregation of unfolded MDH in a concentration-dependent manner. Stoichiometric ratios of ClpB-B1/2A hexamers to MDH monomers kept less than 75% of [3H]MDH in a soluble state (Fig. 1A). The “holder” activity of ClpB-B1/2A was high, since equimolar concentrations of the E. coli Hsp 40 chaperone DnaJ, known as an efficient “holder” chaperone, prevented MDH aggregation to similar degrees. Notably, while protecting MDH from aggregation at 47 °C, ClpB-B1/2A did not support MDH reactivation upon subsequent incubation at 30 °C (data not shown).

Next the nucleotide dependence of ClpB substrate interaction was investigated. No prevention of MDH aggregation by ClpB-B1/2A was observed in the absence of nucleotide or the presence of ADP (2 mM). Consistently, ClpB wild type exhibited partial “holder” activity only in the presence of ATP, but not with ATP (Fig. 1B), demonstrating that association of ClpB with protein substrates occurs in the ATP-bound state. The disaggregating activity of ClpB strictly depends on its ability to form hexamers. ClpB hexamerization is ATP-dependent, however, oligomers can also be formed in low salt buffers (18, 35). We tested whether stabilization of ClpB oligomers in low salt buffer (buffer A lacking KCl) is sufficient to prevent MDH aggregation. In the absence of nucleotide, ClpB-B1/2A did not exhibit any “holder” activity in low salt buffer, while addition of ATP prevented MDH from aggregation (Fig. 1B, data not shown), confirming the need of an ATP-bound conformation, rather than relying only on a hexameric form, for efficient substrate interaction.

We also investigated whether mixed oligomers of ClpB wild type and ClpB-B1/2A are able to prevent MDH aggregation. Firstly, the ability of such mixtures to solubilize MDH aggregates and to hydrolyze ATP was tested. Surprisingly, addition of equimolar concentrations of ClpB-B1/2A, which is deficient in ATP-hydrolysis, to ClpB wild type increased 3-fold the specific ATPase activity (0.068/s compared with 0.021/s), implying the formation of mixed oligomers. While not inhibiting ATP hydrolysis, ClpB-B1/2A completely inhibited solubilization of MDH aggregates by ClpB wild type (plus KJE) at a 1:1 ratio (data not shown). Thus on the one hand ClpB-B1/2A exhibited a strong dominant negative effect on the disaggregating activity of ClpB wild type. On the other hand, such mixtures equally lost the ability to protect MDH from heat-induced aggregation, which is consistent with the determined increased ATPase activity of the mixed oligomers (Fig. 1B). Thereby ClpB wild type also exhibited a strong dominant negative phenotype on the ability of ClpB-B1/2A to associate with substrates in a stable manner.
We next asked, whether the inhibition of ATP-hydrolysis in one AAA domain of ClpB is sufficient to allow stable substrate interaction. The single Walker B mutants ClpB-E279A and ClpB-E678A (termed B1A and B2A, respectively) eliminate the ATPase activity of the corresponding AAA domain (16). Neither ClpB-B1A, nor ClpB-B2A could protect heat-denatured MDH from aggregation (Fig. 1C), showing that both AAA domains need to be frozen in the ATP state to stabilize ClpB substrate interaction. Finally the substrate interaction of other ClpB variants carrying mutations in conserved motifs was probed. Walker A (K212A, K611A, K212A/K611A), sensor 2 (R332A, R756A) or "arginine fingers" (R332A, R756A) mutants did not prevent MDH aggregation (data not shown). Only a double Walker B deletion variant missing the N domain (termed ΔN ClpB-B1/2A) was as active as the corresponding full-length version, confirming the exclusive substrate binding characteristics of ClpB-B1/2A.

In a complementary approach we followed the aggregation kinetics of luciferase at 43 °C in the absence or presence of ClpB variants. The formation of luciferase aggregates was detected as an increase in sample turbidity during incubation at 43 °C. In summary we obtained identical results compared to the MDH data. A 2-fold excess of ClpB-B1/2A hexamers to luciferase was sufficient to completely suppress luciferase aggregation in the presence of 2 mM ATP (Fig. 2A). In the absence of nucleotide or the presence of ADP (2 mM) ClpB-B1/2A did not protect luciferase from aggregation and similar aggregation kinetics as for luciferase alone (without ATP or with ADP) were recorded (Fig. 2A, data not shown). ClpB wild type exhibited only in the presence of ATP some prevention of aggregation activity, while single Walker B variants ClpB-B1A and ClpB-B2A had very minor activity (Fig. 2B). Deletion of the N-terminal domain of ClpB did not affect the "holder" activity of ClpB-B1/2A, as shown for MDH.

ClpB-B1/2A Stably Associates with Protein Substrates—The noticed "holder" activity of ClpB-B1/2A can likely be attributed to a stabilized substrate interaction of this ClpB variant. We tested for the formation of stable complexes with protein substrates by size exclusion chromatography. α-casein and TrfA, a dimeric protein, which is monomerized by concerted action of ClpB and KJE (23), were chosen as soluble substrates. TrfA was labeled in vivo with l-[35S]methionine and eluted as a dimeric protein in gel filtration runs (Fig. 3A). Addition of ClpB-B1/2A (6 μM) and ATP or ATPγS (2 mM) caused a shift in
the TrfA (2 μM) elution profile, corresponding in size to a complex of hexameric ClpB-B1/2A and TrfA (Fig. 3A, data not shown). We calculated, based on the determined fractions of complexed and free \(^{35}\)S/TrfA and ClpB-B1/2A (Fig. 3A, B; ATP-dependent complex formation between ClpB-B1/2A and TrfA), that one ClpB-B1/2A hexamer binds one TrfA dimer. When the TrfA concentration was increased to 4 μM, 50% of TrfA was found associated with ClpB-B1/2A, whereas the remaining TrfA eluted as a dimer (data not shown). Given the high stability of TrfA dimers, which need to be actively converted to monomers either by ClpX or ClpB/DnaK, we therefore suggest binding of one TrfA dimer to one ClpB hexamer. Complex formation was ATP-dependent and did not occur in the absence of ATP.
of nucleotide or in the presence of ADP (2 mM). ΔN ClpB-B1/2A also interacted with TrfA in the presence of ATP in a stable manner, confirming that N-domains are not essential for substrate interaction (Fig. 3C). Co-elution of [35S]TrfA with hexameric ClpB was confirmed by monitoring the elution profile of 3H-labeled ClpB-B1/2A in the absence or presence of TrfA. Labeling of ClpB-B1/2A did not affect the oligomeric state or the complex formation with TrfA (data not shown). ClpB-B1/2A eluted as a dimer, if ATP was omitted from the running buffer (Fig. 3D). Binding of TrfA caused stabilization of the ClpB-B1/2A hexamer, indicating an interdependency between ClpB oligomerization and substrate binding; while ATP-dependent hexamerization of ClpB is essential for stable substrate interaction, binding of substrates in turn causes stabilization of ClpB oligomers.

ClpB wild type, as well as the single Walker B mutants ClpB-B1A and ClpB-B2A were not able to form stable complexes with TrfA, confirming that inhibition of ATP hydrolysis in both AAA domains is essential for stabilizing the interaction with substrates. ATPγS-bound ClpB wild type caused only a slight shift in the elution profile of TrfA, reflecting a stabilized but still transient interaction with the substrate (Fig. 3B). The reduced stability of such complexes is consistent with the lower activity of ATPγS-bound ClpB wild type in preventing MDH and luciferase aggregation (Figs. 1B and 2B). Since complex stability of ClpB-B1/2A and TrfA was not affected by ATPγS (data not shown), we suggest that ClpB wild type can slowly hydrolyze the ATP analogue.

We also tested whether GroEL-D87K, another chaperone trap mutant, recognizes TrfA. This GroEL variant binds but does not hydrolyze ATP and consequently does not release bound substrates (4). Surprisingly, no complex formation between TrfA and the GroEL trap was observed (Fig. 3B).

In an additional approach the binding of ClpB wild type and mutants (6 μM) to 3H-labeled α-casein (1 μM) was monitored and similar results were obtained. Stable complexes were only revealed for ClpB-B1/2A and ΔN ClpB-B1/2A in the presence of ATP, but not for ClpB wild type or the mutants ClpB-B1A and ClpB-B2A (data not shown). Binding of α-casein to mixtures of ClpB wild type and ClpB-B1/2A (6 μM each) was strongly reduced, compared with the association of α-casein with equimolar concentrations of ClpB-B1/2A only (data not shown), confirming the dominant negative effect of ClpB wild type on substrate binding by ClpB-B1/2A. As control we also tested for complex formation between ClpB-B1/2A and bovine serum albumin (5 μM). ClpB-B1/2A did not form complexes with bovine serum albumin and thus exhibited specificity toward its protein substrates (data not shown).

ClpB-B1/2A Prevents the Interaction of DnaK with the Model Substrate TrfA—Since ClpB-B1/2A exhibited a strongly stabilized interaction with protein substrates, we used this ClpB variant as a tool to determine the order of action of ClpB and the DnaK chaperone system during disaggregation reactions. Does ClpB-B1/2A have the potential to prevent binding of DnaK to substrates, thereby indicating that ClpB is acting first during a disaggregation reaction? As a first model substrate we chose dimeric TrfA, which is monomerized only by cooperative action of ClpB and KJE and thus can be described as a protein aggregate of minimal size. First we tested for complex formation between DnaK/DnaJ and TrfA by size exclusion chromatography. DnaK/DnaJ (2 μM DnaK, 1 μM DnaJ) did not show a stable interaction with TrfA (2 μM) during gel filtration and did not prevent association of TrfA with ClpB-B1/2A (data not shown). As an alternative method to monitor the interaction of DnaK with TrfA we tested whether TrfA can stimulate the ATPase activity of DnaK in single turnover experiments (Fig. 4).

The ATPase activity of DnaK (0.5 μM) was stimulated 200-fold by TrfA (1 μM) in the additional presence of DnaJ (0.2 μM). The presence of ClpB-B1/2A (3 μM) largely prevented interaction of TrfA with DnaK, as revealed by a decrease in the TrfA-dependent stimulation of the DnaK ATPase activity (Fig. 4A). This inhibitory effect was completely compensated by increased substrate levels (2 μM TrfA), indicating that one ClpB-B1/2A oligomer interacts with one TrfA dimer. The inhibition of the DnaK ATPase activity by ClpB-B1/2A was dependent on ATP and was not observed in the absence of nucleotides or the presence of ADP (Fig. 4B). Analysis of ClpB variants revealed that the inhibitory effect directly correlated with the ability of the mutants to associate with protein substrates in a stable manner. Thus ΔN ClpB-B1/2A blocked the TrfA-dependent stimulation of the DnaK ATPase activity, while ClpB wild type and single Walker B mutants did not (Fig. 4C).

Notably ClpB-B1/2A alone also slightly stimulated the ATPase activity of DnaK and thus can be recognized by DnaK as substrate (Fig. 4A). The stimulation of the ATPase activity of DnaK by ClpB-B1/2A was independent of nucleotides and was also observed for ClpB wild type or single Walker B variants (data not shown). We therefore assume that recognition of ClpB by DnaK as substrate is rather unspecific and not a functional part of the disaggregation reaction.
ClpB-B1/2A Inhibits the KJE-mediated Dissociation of Soluble sHsp/Substrate Complexes—Soluble complexes of sHsps and protein substrates represent small protein aggregates, which can be actively dissociated by KJE independent of ClpB. However, this disaggregation reaction is stimulated by ClpB wild type and can therefore be used as an alternative system to determine the order of ClpB and KJE action on protein substrates. We followed the refolding of unfolded MDH bound to the small heat shock protein Hsp16.6 from Synechocystis sp. PCC6803 in the presence of ClpB wild type or ClpB-B1/2A. ClpB wild type increased the KJE-dependent MDH refolding rates by 2-fold (9 nM MDH/min compared with 4 nM MDH/min by KJE alone), while addition of ClpB-B1/2A did not allow MDH refolding (Fig. 5A, data not shown). In contrast, addition of ClpB-B1/2A strongly inhibited MDH reactivation by KJE and decreased the refolding rates by 5-fold (0.75 nM MDH/min). We additionally used the GroEL-D87K mutant, which also efficiently blocked MDH refolding by KJE (Fig. 5A). Furthermore, both chaperone trap mutants immediately inhibited or slowed down the ongoing KJE-dependent refolding process in order-of-addition experiments (Fig. 5C). The dominant negative effect of ClpB variants on substrate refolding directly correlated with their ability to associate with substrates in a stable manner. Mixtures of ClpB wild type and ClpB-B1/2A neither inhibited nor stimulated MDH refolding, consistent with the noticed lack of disaggregation and “holder” activities of such mixtures (Fig. 5A). ΔN ClpB-B1/2A exhibited the same dominant negative phenotype as ClpB-B1/2A, while the single Walker B variants ClpB-B1A and ClpB-B2A slightly stimulated the KJE-dependent refolding process of MDH (Fig. 5B).

Although ClpB-B1/2A and GroEL-D87K both inhibited MDH refolding from sHsp/MDH complexes by KJE, it is unclear whether they act on the same stages of MDH reactivation. To dissect mechanistically the dominant negative effects of ClpB-B1/2A and GroEL-D87K on the MDH reactivation process from Hsp16.6/MDH complexes, MDH was labeled with [3H], and the [3H]MDH species, generated during the refolding process, were analyzed by gel filtration chromatography. The addition of KJE caused dissociation of the stable sHsp/MDH complexes and resulted in the formation of monomeric and dimeric MDH molecules, respectively (Fig. 6A). Addition of GroEL-D87K or ClpB-B1/2A alone to the Hsp16.6/MDH complexes did not cause complex dissociation and no transfer of [3H]MDH to the GroEL or ClpB trap mutant was observed (data not shown).

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Fig. 5. ClpB-B1/2A inhibits the KJE-dependent refolding of MDH bound to sHsps. A, 1 μM MDH was heat-denatured in the presence of 4 μM Hsp16.6 resulting in the formation of soluble sHsp/MDH complexes. MDH refolding was started by addition of chaperones as indicated at 30 °C (KJE: 1 μM DnaK, 0.2 μM DnaJ, 0.1 μM GrpE, 7 μM GroEL-D87K; 6 μM ClpB wild type; 6 μM ClpB-B1/2A; and MDH activities were determined at the indicated time points. The enzymatic activity of native MDH was set at 100%. B, MDH was denatured in the presence of Hsp16.6 as described above. MDH refolding was initiated by addition of E. coli chaperones as indicated at 30 °C (KJE: see above; ClpB-B1A, ClpB-B2A, ΔN ClpB-B1/2A: 6 μM each). MDH activities were determined at the indicated time points. C, refolding of MDH from sHsp/MDH complexes was initiated by addition of KJE (see above) at 30 °C. 6 μM ClpB-B1/2A or 7 μM GroEL-D87K were added after 60 min (see arrow) and MDH activities were determined at the indicated time points.
When GroEL-D87K and KJE were added to the Hsp16.6/MDH complexes all MDH molecules were found associated with the GroEL mutant after 120 min incubation time (Fig. 6A). Importantly, GroEL-D87K did not affect the KJE-dependent dissociation of sHsp/MDH complexes, as can be seen from the disappearance of [3H]MDH from the high molecular weight fraction. GroEL-D87K rather inhibited MDH refolding by binding to already extracted but still unfolded MDH molecules. In contrast, when ClpB-B1/2A and KJE were added to the complexes, no complex dissociation was observed (Fig. 6A). Thus ClpB-B1/2A, in contrast to GroEL-D87K, blocked already the first step of the MDH refolding process by inhibiting the KJE-dependent dissociation of sHsp/MDH complexes.

Additionally we followed the fate of [3H]MDH species during order-of-addition experiments. Dissociation of sHsp/MDH complexes was initiated by KJE and ClpB-B1/2A was added after 55 min to the ongoing disaggregation reaction (Fig. 6B). The presence of ClpB-B1/2A immediately blocked further complex dissociation by KJE and additionally prevented conversion of MDH monomers to dimers. Importantly, ClpB-B1/2A specifically interacted with monomeric, unfolded MDH, but not with dimeric MDH species. Consistently, ClpB-B1/2A also inhibited further reactivation of MDH by KJE when added after 55 min to an ongoing refolding reaction (Fig. 5C). This inhibitory effect can be explained by binding of ClpB-B1/2A to already extracted, but still unfolded MDH molecules, which existed at the time point of ClpB-B1/2A addition. A fraction of the monomeric MDH molecules was isolated in complex with hexameric ClpB-B1/2A, while another fraction was eluting as a high molecular weight complex, presumably representing small protein aggregates (Fig. 6B). We assume that dissociation of some MDH molecules from ClpB-B1/2A during the gel filtration run occurred, resulting in MDH aggregation.

These findings suggest that ClpB-B1/2A might outcompete DnaK for binding to sHsp/MDH complexes due to its stabilized substrate interaction. We tested whether increased KJE levels can overcome the dominant negative effects of ClpB-B1/2A on MDH refolding. Higher levels of KJE indeed increased the
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MDH refolding rates 2.5-fold in the additional presence of ClpB-B1/2A, without changing the MDH refolding rates in the absence of ClpB-B1/2A (Fig. 7). However, even 4-fold higher levels of KJE (compared with the KJE amount present in all standard MDH refolding assays) could not entirely compensate the inhibitory effect of ClpB-B1/2A on KJE-dependent substrate refolding. MDH refolding was started by addition of increasing KJE concentrations (1–4 μM DnaK, 0.2–0.8 μM DnaJ, 0.1–0.4 μM GrpE) at 30 °C in the absence or presence of 0.6 μM ClpB-B1/2A. MDH activities were determined and refolding rates were calculated from the linear increase of substrate activities.

In a complementary approach we tested whether association of ClpB-B1/2A with protein substrates was demonstrated by its ability to hydrolyze ATP (16, 19, 36–38). We could show that ClpB-B1/2A partially inhibits the refolding of aggregated MDH by ClpC/MecA, indicating that ClpB-B1/2A is not affecting MDH reactivation by inhibiting KJE activity directly.

ClpB-B1/2A Partially Inhibits the Refolding of Aggregated MDH by ClpC/MecA—In a complementary approach we tested whether ClpB-B1/2A can also affect the solubilization of protein aggregates by other chaperone system. As an alternative disaggregation system we used the Hsp100 protein ClpC from Bacillus subtilis, which can rescue aggregated proteins in cooperation with its adaptor protein MecA (29). Refolding of heat-aggregated MDH was followed by ClpC/MecA alone and in the additional presence of ClpB wild type and variants (Fig. 8). Reactivation of MDH by ClpC/MecA was partially inhibited by equimolar concentrations of ClpB-B1/2A and its deletion variant missing the N domain (Fig. 8). Since ClpB wild type and single Walker B mutants did not affect ClpC/MecA activity, the noticed inhibitory effects of ClpB-B1/2A could again be attributed to its stabilized substrate interaction. These findings confirm that ClpB-B1/2A can inhibit disaggregation reactions by competing with other chaperones for substrate binding.

ClpB-B1/2A Binds to Heat-aggregated Proteins in Vivo—Finally the in vivo relevance of the described findings was investigated. We tested whether association of ClpB-B1/2A with substrates and linked dominant negative effects can be also detected in E. coli cells. We monitored the solubilization of protein aggregates, generated by temperature upshift to 45 °C, in ΔclpB mutant cells harboring plasmid-encoded IPTG regulated clpB alleles (encoding ClpB wild type (WT) and ClpB-B1/2A). Complementation of the ΔclpB phenotype was performed in presence of 10 μM IPTG, leading to ClpB levels typical for heat-treated cells (data not shown). Notably strong overproduction of ClpB-B1/2A in the presence of 250 μM IPTG was toxic, while identical levels of ClpB wild type did not affect cell viability (data not shown). Removal of protein aggregates was strongly impaired in ΔclpB mutant cells, consistent with published data (10). However, we noticed that a slow dissociation of the sHsps IbpA/B from protein aggregates was still detectable in ΔclpB. Such dissociation was no longer observable in ΔdnaK mutant cells, indicating a disaggregation activity of DnaK independent of ClpB. Plasmid expressed ClpB wild type restored protein disaggregation in ΔclpB mutant cells and consequently also facilitated dissociation of sHsps from aggregated proteins.

FIG. 8. ClpB-B1/2A partially inhibits the refolding of aggregated MDH by ClpC/MecA. 1 μM MDH was heat-denatured at 47 °C for 30 min. Refolding of aggregated MDH was initiated by the addition of ClpC and MecA (1 μM each) and an ATP-regenerating system. ClpC/MecA-dependent reactivation of MDH was also followed in the additional presence of 1 μM ClpB wild type and variants as indicated. MDH activities were determined and refolding rates were calculated from the linear increase of substrate activities and set as 100% for ClpC/MecA alone.

DISCUSSION

This study characterizes a variant of the AAA+ chaperone ClpB with mutational alterations in the Walker B motif of both AAA domains (E279A/E678A). Walker B mutants of AAA proteins can form oligomers and have been shown to bind but not to hydrolyze ATP (16, 19, 36–38). We could show that ClpB-E279A/E678A (also termed ClpB-B1/2A), in contrast to ClpB wild type, interacts with protein substrates in a stable manner, thereby demonstrating for the first time direct ClpB substrate interactions in vitro and in vivo. Complex formation of ClpB-B1/2A with protein substrates was demonstrated by its ability to protect thermolabile proteins from heat-induced aggregation and to form stable complexes with TrfA and α-casein as detected by gel filtration. Substrate interaction was strictly dependent on ATP binding to ClpB-B1/2A and was not detectable in the absence of ATP or the presence of ADP. Interestingly, both AAA domains of ClpB have to be frozen in the ATP-bound state in order to guarantee stable substrate binding. Thus the single Walker B mutants ClpB-E279A and ClpB-E678A, which still exhibit ATPase activity (16), did not prevent the heat-induced aggregation of MDH or luciferase and did not form stable complexes with protein substrates. These data imply

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The AAA domains of ClpB must therefore mediate substrate binding.

Stabilized substrate interaction of other AAA proteins have been observed in the presence of non-hydrolysable ATP analogs (ATPγS, AMP-PNP) or transition state analogs of ATP hydrolysis (ADP-ALF4) (24, 25, 39). ClpB-B1/2A exhibits the same binding characteristics in the presence of ATP. This is of fundamental importance if the consequences of a stabilized ClpB substrate interaction on protein disaggregation reactions are investigated. Solubilization of aggregated proteins is only performed by cooperation of ClpB and the DnaK system, while ClpB has no dissociating activity on its own. In order to determine the order of ClpB and KJE action during a disaggregation reaction, it is essential to manipulate the substrate affinity of ClpB without disturbing substrate interaction and/or activity of the DnaK system. We used the unique properties of ClpB-B1/2A as a tool to dissect the order of ClpB and KJE action in the disaggregation process by performing competition experiments between ClpB-B1/2A and DnaK for substrate binding. Equimolar concentrations of hexameric ClpB-B1/2A efficiently prevented DnaK from interaction with dimeric TrfA. Stable substrate binding was a prerequisite for efficient substrate competition, since ClpB wild type and the single Walker B variants did not block TrfA binding to DnaK. Interestingly, interaction of DnaK with dimeric TrfA does not lead to TrfA monomerization (23) in contrast to RepA dimers, which are dissociated by KJE only (40). We suggest that a productive dissociation of TrfA dimers relies on the initial interaction of TrfA with ClpB. ClpB-B1/2A also prevented DnaK from dissociating soluble sHsp/substrate complexes (which represent small protein aggregates), while ClpB wild type and single Walker B variants stimulated complex dissociation to variable degrees. These data suggest that ClpB and DnaK might compete for binding to aggregated proteins. In case of small aggregates binding of DnaK can lead to the extraction of substrates, whereas the interaction is non-productive for large sized aggregates. Initial binding of ClpB to protein aggregates may allow more efficient resolubilization by KJE, either by changing aggregate structure or by directly transferring substrates to the Hsp70 chaperone.

Interestingly ClpB-B1/2A differed from GroEL-D87K, which also functions as a chaperone trap mutant and associates with protein substrates in a stable manner. In contrast to ClpB-B1/2A the GroEL trap did not prevent dissociation of sHsp/MDH complexes by KJE and acted only on extracted, unfolded MDH molecules. In agreement with these findings GroEL-D87K did not affect dissociation of aggregated MDH by ClpB and KJE but inhibited only the refolding of solubilized MDH. The inability of GroEL-D87K to inhibit dissociation reactions goes along with the noticed lack of dissociation activities of GroEL for protein aggregates. We suggest that differences in substrate recognition are the basis for the different activities of ClpB and GroEL (wild type and trap variants). Thus no or poor binding of GroEL to protein aggregates may explain the absence of a dissociating activity. In agreement with this hypothesis, GroEL D87K did not form a complex with dimeric TrfA, a putative protein aggregate of minimal size.

In summary we characterized a double Walker B variant of the AAA+ chaperone ClpB that associates with protein substrates in a stable, ATP-dependent manner. The unique properties of Walker B variants can be used as a powerful tool for investigating the functions of AAA proteins. We used the ClpB double Walker B mutant as a tool to determine the order of ClpB and KJE action on protein substrates. Walker B variants
may also allow identifying in vivo substrates of AAA proteins and/or interacting adaptor proteins. Walker B mutants are also attractive candidates for crystallization trials. The strong stabilizing effect of protein substrates on the oligomeric state of Walker B variants might offer an additional promising approach to structure determination, which has been impeded so far by the inherent flexibility of AAA proteins.

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