Biochemical Coupling of the Two Nucleotide Binding Domains of ClpB

**COVALENT LINKAGE IS NOT A PREREQUISITE FOR CHAPERONE ACTIVITY**

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ClpB cooperates with the DnaK chaperone system in the reactivation of protein from aggregates and is a member of the ATPases associated with a variety of cellular activities (AAA+) protein family. The underlying disaggregation reaction is dependent on ATP hydrolysis at both AAA cassettes of ClpB but the role of each AAA cassette in the reaction cycle is largely unknown. Here we analyze the activity of the separately expressed and purified nucleotide binding domains of ClpB from *Thermus thermophilus*. The two fragments show different biochemical properties: the first construct is inactive in ATPase activity assays and binds nucleotides weakly, the second construct has a very high ATPase activity and interacts tightly with nucleotides. Both individual fragments have lost their chaperone function and are not able to form large oligomers. When combined in solution, however, the two fragments form a stable heterodimer with oligomerization capacities equivalent to wild-type ClpB. This non-covalent complex regains activity in reactivating protein aggregates in cooperation with the DnaK chaperone system. Upon complex formation the ATPase activity of fragment 2 is reduced to a level similar to wild-type ClpB. Hence functional ClpB can be reassembled from its isolated AAA cassettes showing that covalent linkage of these domains is not a prerequisite for the chaperone activity. The observation that the intrinsically high ATPase activity of AAA2 is suppressed by AAA1 allows a hypothetical assignment of their mechanistic function. Whereas the energy gained upon ATP hydrolysis at the AAA2 is likely to drive a conformational change of the structure of ClpB, AAA1 might function as a regulator of the chaperone cycle.

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9 According to all determined crystal structures, the fold of AAA cassettes (ATPases associated with a variety of cellular activities)4 is conserved (9, 10). AAA cassettes consist of a core nucleotide binding domain and a C-terminal subdomain. The core domain contains the classical Walker motifs characteristic of P-loop ATPases (11, 12), whereas the C-terminal subdomain has a high content of α-helices.

The recently solved crystal structure of the ClpB monomer/trimer from *Tetrahymena thermophila* and sequence comparisons within the Clp protein family reveal that ClpB can be divided into four distinct domains. An N-terminal domain precedes the first AAA cassette, which has an insertion called middle (linker) region. This domain is followed by the second AAA cassette of ClpB. A number of biochemical studies give insight into the structures and functions of the individual domains of ClpB (13). The N-terminal domain, which precedes the first AAA cassette, consists of two 75-amino acid residue long repeats (Fig. 1A). The structure of full-length ClpB shows that the N-terminal domain is rather mobile and does not interact tightly with the first AAA cassette. In the structure of the isolated N-domain of ClpB from *E. coli* a small hydrophobic patch was identified. This area was supposed to be important for interactions with substrates (14). However, the N-domain is dispensable for chaperone activity *in vivo* and *in vitro* (8, 15, 16). In ClpA, the N-terminal domain interacts with an adaptor protein, which influences substrate specificity (17). An adaptor protein of ClpB has not been identified and therefore the function of the N-domain of ClpB is not completely understood.

Both AAA cassettes of ClpB must be functional for chaperone activity (2). Mutations in the Walker A or Walker B motifs lead to a loss of chaperone activity and show a complex pattern of effects on the properties of ClpB. Mutations in the Walker A motif of the first AAA cassette affect the steady state ATPase activity of ClpB from *T. thermophilus* and *E. coli* (15, 18, 19). Additionally the oligomerization capacity is reduced and the chaperone function severely impaired. The mutations in the Walker A motif of the first AAA cassette do not affect the nucleotide binding of ClpB, indicating that the second AAA cassette has a high

4 The abbreviations used are: AAA, ATPases associated with a variety of cellular activities; AAA cassette, ATPase domain of AAA proteins; ClpB, ClpB from *T. thermophilus*; DnaK system, chaperone system consisting of DnaK, DnaJ, and GrpE from *T. thermophilus*; GPDH, glucose-6-phosphate dehydrogenase; Ni-NTA, nickel-nitrilotriacetic acid; LDH, lactate dehydrogenase; MOPS, 4-morpholinopropanesulfonic acid; BisTris, 2-[bis(2-hydroxyethyl)aminio]-2-[hydroxymethyl]propane-1,3-diol; HPLC, high pressure liquid chromatography; AMPPCP, adenosine 5′-[β,γ-methylene]triphosphate.
affinity for nucleotides (18, 19). Interestingly ATP hydrolysis at the first AAA cassette is not essential for oligomerization. Proteins with mutations in the Walker B motif show reduced ATPase activity but still bind nucleotides and do not exhibit assembly defects (15, 19). The importance of the first AAA cassette for oligomerization can be explained with residues of adjacent subunits within the ClpB hexamer interacting with the nucleotide bound to the preceding AAA cassette (13).

A unique feature of ClpB is an insertion in the first AAA cassette. This middle domain formerly also called the link domain is not found in other Clp proteins. It is inserted in a loop of the helical subdomain of the first AAA cassette. In the ClpB structure this insertion forms a long coiled-coil structure composed of two leucine-rich segments (13). The domain protrudes widely from the core of the molecule, has relatively high local flexibility in the structure. The middle region and its flexible conformation were shown to be essential for the chaperone activity of ClpB in vitro (13, 15). Following this insertion the first AAA cassette continues with an α-helix connecting the two AAA cassettes of ClpB.

Mutations in the Walker A motif of the second AAA cassette affect the ATPase activity of ClpB from T. thermophilus, however, their effect on oligomerization is less pronounced than for the first AAA cassette (18, 19). Similar results are published for ClpB from E. coli, but interestingly in Hsp104 from S. cerevisiae the functions of the AAA cassettes seem to be reversed. Here mutations in the first AAA cassette eliminate ATP hydrolysis but do not effect oligomerization, whereas mutations in the P-loop of AAA cassette 2 severely impair oligomerization and reduce ATPase activity (15, 20).

One observation is conserved between the ClpB proteins from different organisms: ClpB variants with mutations in the Walker A motifs of either AAA cassette have severely reduced steady state ATPase activities and are inactive in chaperone activity assays (15, 18, 20). These findings show that for full steady state ATPase activity as well as chaperone function of ClpB cooperativity of the two AAA cassettes is necessary.

In this study we examined the biochemical properties and the interactions of the two nucleotide binding domains of ClpB from T. thermophilus. A comparison of nucleotide binding and ATPase properties of the individual fragments and the chaperone competent reassembled complex indicates that: 1) covalent linkage between the two nucleotide domains of ClpB is not necessary for functional coupling and 2) the presence of NBD1 strongly suppresses an intrinsically high ATPase activity of NBD2, which indicates a tentative assignment for the role of this hydrolysis step.

MATERIALS AND METHODS

Mutagenesis—Constructs for the two fragments (ClpB-(141–519) and ClpB-(519–854)) were produced by PCR-directed mutagenesis using petRS-ClpB as template (18). The PCR products were purified and digested with restriction enzymes NdeI and EcoRI. The DNA fragments were cloned into NdeI-EcoRI-restricted vector pET28a (Novagen) using LB medium at 37 °C and purified as described (25). If not noted otherwise, all protein concentrations refer to monomers.

Gel Filtration Experiments—Oligomerization of the proteins was analyzed by gel filtration experiments at 25 °C. The experiments were performed using a Superdex 200 10/30 column (Amersham Biosciences) with a Waters HPLC system (Waters). ClpB-(141–519), ClpB-(519–854), ClpBΔN, and the complex of ClpB-(141–519)-ClpB-(519–854) were injected (50 μL, 5 mg/mL) with a flow rate of 0.2 ml/min (running buffer: 50 mM Tris/HCl, pH 7.5, 20 mM MgCl₂, 1 mM EDTA, 1 mM dithioerythritol, 10% glycerol, and 1–500 mM KCl). If indicated, nucleotides (1 mM) were added to the running buffer and the protein sample.

Calibration of the gel filtration column was performed using a gel filtration standard (Bio-Rad) and established the exponential relation between the molecular mass and retention time of subsequently analyzed proteins (data not shown). The oligomeric forms of ClpBΔN (80.9 kDa), ClpB-(141–519) (43.2 kDa), and ClpB-(519–854) (38.3 kDa) were defined within the following limits (kDa): ClpBΔN, monomer (M) 70–130, dimer (D) 130–200, trimer (T) 200–290, tetra-hexamer (H) 290–530; ClpB-(141–519), M 30–60, D 60–100, T 100–150, H 150–270; ClpB-(519–854), M 25–55, D 55–90, T 90–140, H 140–250. For analysis of the oligomerization behavior of the complex consisting of ClpB-(141–519)-ClpB-(519–854) the limits of ClpBΔN were used.

Circular Dichroism—CD spectra were recorded (10-fold accumulation) with a Jasco-J710 Spectropolarimeter in 0.02-cm cuvettes at a scan rate of 20 nm/min, 0.2-nm resolution, 1 nm bandwidth, a time constant of 1 s, and a sensitivity of 20 mdeg at 25 °C.

Fluorescence Measurements—Nucleotide affinities were determined in equilibrium titration experiments using a SLM 8100 Photon-counting spectrofluorimeter at 25 °C (18). The relative fluorescence was plotted against the protein concentration and the data were analyzed with the quadratic equation using the program Grafit, version 3.01 (26). The equilibrium displacement of the fluorescent nucleotide by ADP were fitted to a cubic equation (27).

Chaperone-assisted Reactivation of Substrate Proteins—Substrate proteins were denatured in reaction buffer (50 mM MOPS/NaOH, pH 7.5, 150 mM KCl, 10 mM MgCl₂, 5 mM ATP, 2 mM dithioerythritol) and chaperones were added (ClpB variants 0.5 μM, DnaK system: DnaK, 1.6 μM; Dnal, 0.4 μM; GrpE, 0.2 μM) prior to refolding at 55 °C. α-Glucosidase (0.1 μM) from B. stearothermophilus (Sigma) was denatured 10 min at 75 °C (5, 8). α-Glucosidase activity was determined at 40 °C at the indicated time points of the refolding reaction by diluting...
the reaction mixture into the assay solution (50 mM sodium phosphate, 2 mM p-nitrophenyl-β-D-glucopyranoside).

Glucose-6-phosphate dehydrogenase (GPDH) (0.2 mM) from *B. stearothermophilus* (Sigma) was denatured 7.5 min at 75 °C (5). GPDH activity was determined at 40 °C at the indicated time points of the refolding reaction by diluting the reaction mixture into assay buffer (100 mM Tris/HCl, pH 8.8, 40 mM MgCl₂, 3 mM glucose 6-phosphate, 1 mM NADPH, 0.1 mg/ml bovine serum albumin).

LDH from *B. stearothermophilus* (0.2 mM) was incubated 30 min at 80 °C (5, 8). LDH activity was determined at 25 °C at the indicated time points of the refolding reaction by diluting the reaction mixture into assay buffer (25 mM BisTris/HCl, pH 6.5, 50 mM KCl, 10 mM pyruvate, 0.25 mM NADH).

Activity of the native substrate proteins was determined before the denaturation step. Concentrations refer to substrate protein solutions prior to aggregation by heat shock.

**Isothermal Calorimetry**—Isothermal calorimetric titrations were performed with a MicroCal MCS isothermal titration calorimeter (MicroCal LLC, Northampton, MA) to measure the binding affinity of the ClpB fragments. The heat of binding was detected upon the titration of ClpB-(141–519) (90 μM) into a cell containing ClpB-(519–854) (10 μM) with 50 mM HEPES, pH 7.5, 100 mM KCl, and 5 mM MgCl₂ as buffer system. Experiments were done at 45 °C at an injection rate of 0.5 μl/s. 29 injections were performed with a spacing of 240 s between each. The data were analyzed using the manufacturer’s software to calculate the reaction enthalpy, stoichiometry of binding, and association constant.

**Steady State ATPase Measurements**—Steady state ATP hydrolysis was measured with a coupled colorimetric assay (18, 28). ClpB variants (10 μM) were incubated at 25 °C with different concentrations of Mg-ATP in assay buffer (50 mM Tris/HCl, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 2 mM EDTA, 2 mM dithioerythritol, 0.4 mM phosphoenolpyruvate, 0.5 mM NADH, 20 μg/ml LDH, 50 μg/ml pyruvate kinase). The observed rate constants were determined from the decrease of p-nitrophenyl-D-glucopyranoside. The UV CD spectra of ClpB-(141–519) (dotted line), ClpB-(519–854) (dashed line), and ClpBΔN (solid line) are shown. The circular dichroism is given as mean molar ellipticity per amino acid (θ).

**RESULTS**

Design of ClpB Constructs—ClpB fragments were constructed based on sequence alignments of ClpB proteins from different organisms and the crystal structure of ClpB (Fig. 1A) (13). The constructs used in this study are shown in Fig. 1B. For stability reasons, the N-terminal domain of ClpB, which is dispensable for function, was not included in the construct coding for the first AAA cassette. This construct started at position 141, where an alternative start codon is present in the *E. coli*
mRNA being responsible for the natural occurrence of ClpBΔN (29, 30). Because the ClpB-specific middle region inserts within the first AAA cassette it was included in the first construct. The C-terminal end of the first construct was behind the insertion of the coiled-coil domain and close to the beginning of the second AAA cassette. We introduced a stop codon after position 519. Hence the last 15 amino acids (519–534) of the first AAA cassette were not present in this construct. The second construct started at position 519 and ended with the naturally used stop codon after amino acid 854. The last 15 amino acids (519–535) of the first AAA cassettes as seen in the structure were included in the construct for the second AAA cassette. The additional N-terminal amino acids from the first AAA cassette allowed this construct to fold in its native structure and facilitated interdomain communication.

Both proteins were recombinantly expressed in E. coli and purified by affinity chromatography and gel filtration. The correct folding of the constructs was assessed by CD spectroscopy (Fig. 1C). The CD spectra of both fragments had local minima at 208 and 222 nm typical of folded proteins with a relatively high content of α-helices. The first construct contains a coiled-coil domain and shows a typical strong peak at 208 nm. This is in agreement with the secondary structure of ClpB observed in the crystal structure and shows that the independently expressed ClpB fragments are folded correctly, which is a prerequisite for the functional characterization of the constructs.

**Oligomerization of the ClpB Variants**—ClpB can form hexamers in the presence of ATP and this oligomerization is essential for the chaperone function of the protein (31). The oligomeric forms of the constructs were analyzed by gel filtration experiments with different ionic strengths of the running buffer in the presence of ADP or ATP. Analysis of ClpB-(141–519) under the tested conditions revealed that this fragment does not form oligomers (Fig. 2A). The predominant form of this protein was the monomer with minor amounts of dimers present at lower ionic strength. Nucleotides present in the buffer did not affect the oligomerization of ClpB-(141–519). Because a ClpB construct lacking only the C-terminal subdomain of the second AAA cassette (ClpB-(1–762)) is also monomeric (data not shown) we can exclude that the missing N terminus or the last 10 amino acid residues of the first fragment (constituting an α-helix) are responsible for lack of oligomerization.

The oligomerization of fragment 2 (ClpB-(519–854)) was examined under identical conditions (Fig. 2B). At low ionic strength monomers and dimers but no higher oligomeric forms were present. ATP stabilized the dimeric form, whereas ADP and higher ionic strengths shifted the equilibrium toward the monomeric state.

A mixture of equimolar amounts of the two ClpB fragments showed a different oligomerization behavior compared with the individual constructs (Fig. 2C). No peaks corresponding to the individual proteins were detectable in the elution profile to significant amounts. Instead, a heterodimeric complex of ClpB-(141–519) and ClpB-(519–854) was formed. In the presence of high salt concentrations and ADP the monomer of this heterodimeric complex was the main oligomeric species. Lower ionic strength and ATP stabilized higher oligomeric forms, in the presence of ATP and 1 mM KCl hexameric forms of the complex were detected. Hence, the complex can form oligomers in contrast to its constituents ClpB-(141–519) and ClpB-(519–854) and the effect of nucleotides and ionic strength on the oligomerization behavior of the complex is similar to ClpB wild-type (18). This complex was stable, which is reflected by the fact that dissociation into its individual components was not observed.

**FIGURE 2. Oligomerization of the ClpB variants tested.** The oligomerization of the ClpB variants (50 μM) was analyzed by gel filtration experiments. Thin lines represent the boundaries set between monomers (M), dimers (D), trimers (T), and higher oligomers up to hexamers (H). ClpB-(141–519) (A), ClpB-(519–854) (B), the mixture of ClpB-(141–519) and ClpB-(519–854) (C), and ClpBΔN (D) were analyzed with nucleotides and different salt concentrations. In C the boundaries refer to the protein complex not the individual ClpB fragments. ATP, 1 mM KCl (black); ATP, 200 mM KCl (blue); ATP, 500 mM KCl (cyan). ADP, 1 mM KCl (yellow); ADP, 200 mM KCl (magenta); ADP, 500 mM KCl (red).
The oligomerization behavior of the complex was compared with the behavior of ClpB/H9004N (Fig. 2D). This ClpB variant is similar in size to the complex formed by the two fragments, is functional in chaperone activity assays, and has biochemical properties similar to ClpB wild-type (8). Compared with ClpB/H9004N the complex of ClpB-(141–519)-ClpB-(519–854) shows a similar oligomerization behavior. Only at low salt concentration in the presence of ADP were less hexamers of heterodimers of the complex detected compared with ClpB/H9004N.

In summary, these gel filtration experiments demonstrate that the two ClpB fragments form a stable protein complex. This complex did not dissociate in the presence of nucleotides and underwent nucleotide-dependent oligomerization, whereas the individual fragments showed only marginal oligomerization capacities and were present mainly as monomers.

Affinity of the Two Protein Fragments and Binding Stoichiometry—To determine the affinity of the fragments, isothermal calorimetry titrations were performed and the result is shown in Fig. 3. The addition of ClpB-(141–519) to a solution containing ClpB-(519–854) gave a pronounced heat (ITC—) signal in the experiment. Data analysis with a simple binding model indicated a dissociation constant of 7 nM, which is at the lower limit of detection. This strong interaction is driven by a change in enthalpy (ΔH = −38.7 kcal/mol) upon complex formation, whereas the entropic contribution is highly unfavorable with −0.093 kcal/(mol K) (−27 kcal/mol at 45 °C). The obtained binding stoichiometry of 0.8:1 indicates within experimental error of protein concentration determination a 1:1 ratio of the fragments in the complex. A 1:1 stoichiometry was also supported by gel filtration experiments where equimolar amounts of the fragments were needed for quantitative complex formation. Analysis of the fractions corresponding to the peak of the heterodimeric complex by SDS-gel electrophoresis confirmed the presence of both fragments in the complex (data not shown).

**ATPase Activity and Nucleotide Binding of the ClpB Variants**—In the following experiments we studied the ATPase activities of the ClpB fragments. First, the steady state ATPase activities of the proteins were performed and the result is shown in Fig. 4. The observed rates of ATP hydrolysis (k) were plotted against the ATP concentration (Fig. 4A). If interactions of the two nucleotide binding sites are essential for the ATPase activity, no steady state ATPase activity of the isolated protein fragments should be detectable.

In fact a very low ATPase activity was observed for the first construct even at high substrate concentrations. This defect is not caused by the
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missing helix at the C terminus of the protein. A longer ClpB fragment (1–762), which has a full-length first AAA cassette is also inactive in all ATPase activity assays (data not shown).

The second fragment, however, was highly active in the steady state measurements. As the plot of the observed rate constants against ATP concentrations showed a sigmoidal dependence on ATP concentration the data were analyzed with the Hill equation describing cooperative behavior. ClpB-(519–854) hydrolyzed ATP with a $k_{cat}$ of 6.9 ± 0.2 min$^{-1}$, which is higher than the reported $k_{cat}$ value of ClpB wild-type (8, 18). The determined Hill coefficient of 2.08 ± 0.21 ($K_H = 760 ± 40 \mu M$) indicates the interaction of at least two active sites. This Hill coefficient can be explained with a dimeric protein form active in ATP hydrolysis. Consistently this oligomeric form of ClpB-(519–854) was also detected in gel filtration experiments (Fig. 2A).

The mixture of ClpB-(141–519) and ClpB-(519–854) showed an allosteric ATPase activity. The activity reached a level similar to the isolated, second fragment at the highest substrate concentration used. However, the activity was low up to 1 mM ATP and increased only at higher ATP concentrations. Analysis with the Hill equation (see "Materials and Methods") yielded a $k_{cat}$ value of 7.6 ± 0.1 min$^{-1}$ and a Hill coefficient of 3.3 ± 0.1 ($K_H = 2.3 ± 0.04$ mM). The changed activity profile of the mixture compared with ClpB-(519–854) is another indication of complex formation, confirming the observations from gel filtration experiments. This complex formation is coupled with a change in the cooperativity of the ATPase function. The maximal ATPase activities of ClpB-(519–854) and the complex are in the range of the $k_{cat}$ value of ClpBΔN (7.5 ± 0.1 min$^{-1}$), which was again used as the reference protein. The Hill coefficient determined for ClpBΔN in this experiment was 2.05 ± 0.08, which is slightly lower than previously reported (8).

Differences in the ATPase activities of the studied ClpB variants were also revealed under single turnover conditions. The percentage of ATP hydrolyzed by the proteins was determined at the indicated time points and plotted against the incubation time. The results are summarized in Fig. 4B. As observed under steady state conditions the first fragment was severely restricted in its capacity to hydrolyze ATP. The ATPase activity of the complex consisting of ClpB-(141–519) and ClpB-(519–854) was higher and analysis with a single exponential equation yielded a rate constant of $k = 2.2 × 10^{-2}$ min$^{-1}$. With a rate constant of $k = 3.2 × 10^{-2}$ min$^{-1}$ ClpBΔN showed a similar single turnover ATPase activity. These values are close to the value reported for the single turnover ATPase of ClpB wild-type with $k = 1.8 × 10^{-2}$ min$^{-1}$ (18).

Remarkably the ATPase activity of ClpB-(519–854) was much higher than the activity of the other constructs, after 2 min of incubation more than 90% of the ATP was hydrolyzed. To determine the rate constant, rapid quenching experiments had to be performed (see "Materials and Methods"). The percentage of hydrolyzed ATP plotted against incubation time is shown in Fig. 4C. To describe the experimentally determined values a double exponential equation had to be used. A fast initial reaction with $k = 3.5 ± 0.8$ s$^{-1}$ (210 min$^{-1}$) and a second slower reaction with $k = 0.13 ± 0.04$ s$^{-1}$ were determined. Both reactions had similar amplitudes of 43 ± 5 and 41 ± 5%. The second rate constant of 0.13 s$^{-1}$ (7.8 min$^{-1}$) is in the range of the determined steady state ATPase activity of the protein. These results show that the isolated second AAA cassette of ClpB catalyzes a fast ATP hydrolysis reaction. The determined rate constants under single turnover conditions are much higher compared with ClpBΔN, the complex of ClpB-(141–519)-ClpB-(519–854), or ClpB wild-type (factor 250–400).

In addition, the equilibrium binding affinities of the constructs for nucleotides were examined. Binding of the fluorescent MANT-ADP was analyzed by equilibrium binding titrations; the binding of ADP was studied by displacement experiments (18). For the first fragment only a weak binding signal was detectable giving a dissociation constant of above 50 μM. Using ClpB-(1–762) as a control, low affinity binding ($K_d \approx 45 \mu M$) is also observed confirming that the first AAA cassette has a low affinity for nucleotides. Because of the limitations of the titration experiments extremely low affinities cannot be determined precisely, but it seems plausible that binding is not severely affected by the missing C-terminal helix of the AAA cassette. The second fragment bound the modified nucleotide (MANT-ADP) with high affinity ($K_d \approx 0.07 \mu M$). The $K_d$ values are similar to those observed with ClpB wild-type and show that the second AAA cassette is the high affinity binding site as proposed (18, 19).

Two more constructs were characterized during our experiments. A ClpB construct lacking the middle domain (ClpBΔM = ClpBΔ-(394–532)) and a construct starting with the middle region (ClpB-(394–854)). Both constructs had a very high ATPase activity under single turnover and steady state conditions. They bound nucleotides with high affinity and therefore their biochemical properties are similar to the second fragment (ClpB-(519–854)) used in this study (data not shown).

These experiments demonstrate that the nucleotide binding domains of ClpB have large differences in their biochemical properties. The first fragment is deficient in ATP hydrolysis and binds nucleotides very weakly; the second fragment is more active than the wild-type protein in the ATPase activity measurements and has a high affinity for nucleotides. Upon reconstitution of ClpB out of the fragments the ATPase activity is reduced compared with the isolated second NBD. Under steady state conditions the maximal activity is reached at higher substrate concentrations and the single turnover ATPase activity is lowered to the activity of ClpBΔN.

**Chaperone Activities of the ClpB Fragments and the Protein Complex**—The chaperone activity of ClpB is dependent on two functional AAA cassettes and the ability to form hexamers (18, 31, 32). We used three model substrates for the ClpB-DnaK chaperone network to test the chaperone activity of the ClpB fragments and the protein complex (Fig. 5). Without chaperones only very small amounts of substrate activity were detected during the refolding period (data not shown).

α-Glucosidase was reactivated by the chaperones after denaturation by heat shock (Fig. 5A) (5, 8). ClpBΔN was able to refold 28% of the denatured substrate in cooperation with the DnaK system, which is in agreement with the previously reported refolding capacity of ClpBΔN with this substrate (8). The complex of ClpB-(141–519)-ClpB-(519–854) was functional in this assay, reactivating 28% of the native α-glucosidase activity. Therefore, the chaperone activity of the reconstituted complex in cooperation with the DnaK system was not distinguishable from the activity of ClpBΔN. The fragments alone were inactive in this functional assay. In these reactions only the α-glucosidase activity rescued by the DnaK chaperone system (2–3%) was observed.

Heat-denatured GPDH was used as an alternative substrate for the chaperone network (Fig. 5B) (5). After 90 min of incubation at the permissive temperature for refolding, 50% of the native activity was restored by ClpBΔN in cooperation with the DnaK system. Again no difference between the chaperone activities of ClpBΔN and the complex of ClpB-(141–519)-ClpB-(519–854) was detected. In this experiment the individual fragments were inactive, allowing only the DnaK system to refold up to 10% of the GPDH activity.

As a third substrate, LDH was denatured for 30 min at 80 °C prior to refolding (Fig. 5C). After incubation with ClpBΔN and the DnaK system at the refolding temperature, 40% of the enzyme activity was reactivated. The complex of ClpB-(141–519)-ClpB-(519–854) was able to...
restore 26% of the LDH activity, corresponding to ~65% of the activity rescued by ClpBΔN. As observed before the ClpB fragments were not able to reactivate denatured substrates in cooperation with the DnaK system. In these reactions only the DnaK system was active and reactivated less than 5% of the native substrate activity.

The use of three substrate proteins for the ClpB-DnaK chaperone network allowed a reliable quantification of the chaperone activities. The experiments demonstrate that the complex formed by the two ClpB fragments is functional in vitro. The chaperone activity was identical to that of the second AAA cassette. However, the high ATPase activity of ClpB-(519–854) was unexpected, as full ATPase activity of ClpB wild-type oligomerization is necessary. In addition, both AAA cassettes must be functional and interact in a complex manner to show positive cooperativity in the full-length protein (2, 16, 18).

In ClpB from E. coli, both individually studied AAA cassettes were found to be monomers, inactive in ATPase activity measurements, and did not show any chaperone function (15). The inactivity of the fragments was explained with their deficiency in forming stable hexamers and hexamerization being essential for the allosteric activation of ATPase activity. These differences between the mesophilic and thermophilic proteins may be explained with a different position of the start codon in constructing the second E. coli ClpB AAA cassette. Here position 551 was used corresponding to position 541 in T. thermophilus ClpB. This shortening of the second AAA cassette could have compromised the structural or functional integrity of the second AAA cassette. Alternatively the adaptation toward the higher growth temperature of T. thermophilus compared with the mesophilic E. coli could have led to stronger interactions of the AAA cassettes. However, the high ATPase activity of the second AAA cassette of ClpB from T. thermophilus could indicate fundamental differences in the activity and interactions of AAA cassettes of ClpB from E. coli and T. thermophilus. Further studies on AAA cassettes of ClpB proteins from other organisms might clarify this point.

The AAA cassettes of ClpB from T. thermophilus associated spontaneously to form a high affinity protein complex with an apparent Kₐ of 7 nM. Although mainly monomeric and dimeric, respectively, the heterodimeric protein complex of the two AAA cassettes of ClpB was able to form oligomers. The heterodimeric complex had a similar oligomerization capacity as ClpBΔN or ClpB wild-type. Because the individual domains do not oligomerize, whereas reconstituted ClpB does, both AAA cassettes appear to contribute to higher order oligomerization. This is in agreement with data of E. coli ClpB but different than the well studied AAA protein, N-ethylmaleimide-sensitive fusion protein. In this protein one AAA cassette (D2) has a high affinity for ATP but a low turnover and can form stable hexamers in the presence of ATP. The D2 domain is sufficient and necessary for oligomerization, whereas the second AAA cassette (D1) cannot form oligomers but has a higher ATP turnover (33, 34).

A stable monomer of the heterodimeric complex of ClpB-(141–519) contained the first AAA cassette until the insertion of the middle region and this insertion itself. This construct had only a marginal ATPase activity, bound nucleotides weakly, lost its chaperone activity, and is a monomer according to gel filtration experiments. These results are consistent with results of a longer ClpB construct (ClpB-(1–762)) with a full-length first AAA cassette (data not shown). Therefore the first fragment used here can be regarded to be representative for the complete first AAA cassette of ClpB.

The N terminus of the second fragment (ClpB-(519–854)) was 15 amino acids ahead of the second AAA cassette as evident from the crystal structure of ClpB (13). As the first 10 amino acids are connected to the rest of the protein via a small linker we presume that the biochemical properties resemble those of the second AAA cassette of ClpB. Based on these assumptions an assignment of biochemical functions and properties of both AAA cassettes can be made. The second AAA cassette of ClpB was inactive as a chaperone but highly active in ATPase activity measurements, bound nucleotides strongly, and was able to dimerize. Analysis of the ATPase activity supported the dimer as an active form of this protein. In summary the separately expressed and purified proteins have lost their chaperone activity but they show remarkable differences in other biochemical functions. The high ATPase activity of ClpB-(519–854) was unexpected, as full ATPase activity of ClpB wild-type oligomerization is necessary. In addition, both AAA cassettes must be functional and interact in a complex manner to show positive cooperativity in the full-length protein (2, 16, 18).

In ClpB from E. coli, both individually studied AAA cassettes were found to be monomers, inactive in ATPase activity measurements, and did not show any chaperone function (15). The inactivity of the fragments was explained with their deficiency in forming stable hexamers and hexamerization being essential for the allosteric activation of ATPase activity. These differences between the mesophilic and thermophilic proteins may be explained with a different position of the start codon in constructing the second E. coli ClpB AAA cassette. Here position 551 was used corresponding to position 541 in T. thermophilus ClpB. This shortening of the second AAA cassette could have compromised the structural or functional integrity of the second AAA cassette. Alternatively the adaptation toward the higher growth temperature of T. thermophilus compared with the mesophilic E. coli could have led to stronger interactions of the AAA cassettes. However, the high ATPase activity of the second AAA cassette of ClpB from T. thermophilus could indicate fundamental differences in the activity and interactions of AAA cassettes of ClpB from E. coli and T. thermophilus. Further studies on AAA cassettes of ClpB proteins from other organisms might clarify this point.

The AAA cassettes of ClpB from T. thermophilus associated spontaneously to form a high affinity protein complex with an apparent Kₐ of 7 nM. Although mainly monomeric and dimeric, respectively, the heterodimeric protein complex of the two AAA cassettes of ClpB was able to form oligomers. The heterodimeric complex had a similar oligomerization capacity as ClpBΔN or ClpB wild-type. Because the individual domains do not oligomerize, whereas reconstituted ClpB does, both AAA cassettes appear to contribute to higher order oligomerization. This is in agreement with data of E. coli ClpB but different than the well studied AAA protein, N-ethylmaleimide-sensitive fusion protein. In this protein one AAA cassette (D2) has a high affinity for ATP but a low turnover and can form stable hexamers in the presence of ATP. The D2 domain is sufficient and necessary for oligomerization, whereas the second AAA cassette (D1) cannot form oligomers but has a higher ATP turnover (33, 34).

A stable monomer of the heterodimeric complex of ClpB-(141–519)-

**FIGURE 5. Chaperone activity of ClpB variants in vitro.** The chaperone activities of the ClpB variants were tested in vitro. a-glucosidase (A), GPDH (B), and LDH (C) were used as substrate proteins. Shown are the results for the DnaK system alone (●), ClpBΔN with the DnaK system (○), the complex with the DnaK system (■), ClpB-(141–519) with the DnaK system (□), and ClpB-(519–854) with the DnaK system (△).
ClpB-(519–854) and no individual fragments are seen at high salt conditions in the presence of ADP and ATP. This indicates that in the smallest form of the heterodimeric complex, strong interactions of both AAA cassettes exist and are present during the entire ATPase cycle. The complex formation also affected the observed ATPase activities. The ATPase activities of the heterodimeric complex are substantially lower than the ATPase activities of the isolated second AAA cassette under single turnover and steady state conditions but significantly higher than the activity of the first fragment.

The protein complex showed a single turnover ATPase activity very similar to the reference protein ClpBΔN. Under steady state conditions the cooperativity is changed and the maximum ATPase activity is reached at higher substrate concentration compared with ClpBΔN. The differences between the heterodimeric complex and ClpBΔN can be explained with a slightly reduced interaction in the reassembled complex when compared with the intact full-length protein.

For the reduction of the highly active second AAA cassette, the first AAA cassette itself and the inserted middle domain are necessary. If either one is not present, the second AAA domain remains in its unsuppressed active form. In the crystal structure the interface between the two cassettes is not located close to their nucleotide binding site. Hence, the non-covalent interactions of the AAA cassettes leading to complex formation are apparently sufficient for allosteric regulation and cooperativity of the ATPase activity as observed with ClpB wild-type (18). Although the complex allosteric behavior of the ATPase activity of ClpB can be disturbed easily by point mutations in the nucleotide binding sites of the protein (19, 20), no covalent linkage of the AAA cassettes seems to be necessary for a stable interaction and functional linkage.

In addition, the heterodimeric complex of the two AAA cassettes of ClpB from *T. thermophilus* showed protein disaggregation activity that also indicates that it is able to cooperate with the DnaK chaperone system (36). Accordingly, non-covalent linkage of the AAA cassettes does not only allow allosteric interactions of the nucleotide binding sites but also imparts chaperone activity. ATPase activity measurements and refolding assays are performed in the presence of ATP under low to medium ionic strength. The similarity of the conditions allows the assumption that a similar oligomer is responsible for the activities. Under these conditions the heterodimeric complex of ClpB-(141–519)-ClpB-(519–854) can form oligomers (tetramers to hexamers) as seen in the gel filtration experiments. This indicates that similar to full-length ClpB, a higher order oligomeric, most likely the hexameric form, is the active chaperone component.

But can we assign discrete functions to the AAA cassettes concerning the ATPase/chaperone cycle of ClpB with these results? Clearly both AAA cassettes must be functional individually to allow for chaperone activity and inactivating mutations in the Walker consensus motifs of one of the AAA cassettes leads to a almost complete loss of ATPase activity (19, 20). We showed here that the intrinsically high ATPase activity of the second AAA cassette is substantially reduced upon complex formation with the first AAA cassette. For this reason the first AAA cassette including the middle region is necessary. What is the functional role of this reduction in ATPase activity? As a hypothesis we suggest that ATP hydrolysis at the second AAA cassette of ClpB from *T. thermophilus* could drive a conformational change of the structure of ClpB. This structural rearrangement only takes place if both AAA cassettes form a functional complex, putting a load on the second AAA cassette (37). The ATP hydrolysis at the first AAA domain could then be necessary to complete the reaction cycle, e.g. to dissociate from the substrate or to allow for a new round in the chaperone cycle.

If ClpB functions as a molecular crowbar, this conformational change could be a movement of the coiled-coil middle region in a capture and release mechanism with multiple rounds of substrate binding (13). Alternatively the energy gained upon ATP hydrolysis by the second AAA cassette could be used for a translocation/threading mechanism through the central pore of a ClpB hexamer. In this mechanism the substrate is bound during multiple rounds of ATP hydrolysis and the polypeptide is removed stepwise from the aggregate and translocated. For this mechanism convincing biochemical evidence has occurred recently (38, 39). Based on our kinetic and biochemical data we cannot distinguish between these two mechanisms and a combination of both mechanisms depending on the size of the aggregated substrate proteins (35) is possible.

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