Roles of Individual Domains and Conserved Motifs of the AAA+ Chaperone ClpB in Oligomerization, ATP Hydrolysis, and Chaperone Activity

Axel Mogk*, Christian Schlieker†, Christine Strub§, Wolfgang Rist‡, Jimena Weibezahn§, and Bernd Bukau¶

Received for publication, September 20, 2002, and in revised form, January 23, 2003
Published, JBC Papers in Press, March 6, 2003, DOI 10.1074/jbc.M209686200

ClpB of Escherichia coli is an ATP-dependent ring-forming chaperone that mediates the resolubilization of aggregated proteins in cooperation with the DnaK chaperone system. ClpB belongs to the Hsp100/Clp subfamily of AAA+ proteins and is composed of an N-terminal domain and two AAA-domains that are separated by a "linker" region. Here we present a detailed structure-function analysis of ClpB, dissecting the individual roles of ClpB domains and conserved motifs in oligomerization, ATP hydrolysis, and chaperone activity. Our results show that ClpB oligomerization is strictly dependent on the presence of the C-terminal domain of the second AAA-domain, while ATP binding to the first AAA-domains stabilized the ClpB oligomer. Analysis of mutants of conserved residues in Walker A and B and sensor 2 motifs revealed that both AAA-domains contribute to the basal ATPase activity of ClpB and communicate in a complex manner. Chaperone activity strictly depends on ClpB oligomerization and the presence of a residual ATPase activity. The N-domain is dispensable for oligomerization and for the disaggregating activity in vitro and in vivo. In contrast the presence of the linker region, although not involved in oligomerization, is essential for ClpB chaperone activity.

The Escherichia coli chaperone ClpB belongs to the ring-forming Clp/Hsp100 proteins. Clp/Hsp100 proteins can be classified into two distinct subfamilies. Class I proteins (ClpA and ClpB in E. coli) are composed of two highly conserved nucleotide binding domains (termed ATP-1 and ATP-2), whereas class II proteins (ClpX and HsIu, as representatives of E. coli) contain only a single NBD (homologous to ATP-2) (1). Sequence analysis of the NBDs revealed a significant sequence homology between Clp/Hsp100 and AAA proteins (ATPase associated with a variety of cellular activities), and consequently a new AAA+ superfamily, representing both protein classes, was proposed (2). The structural basis of this superfamily was confirmed by determination of the first Clp/Hsp100 protein structure, HsIu, that showed significant similarity to the AAA proteins N-ethylmaleimide-sensitive fusion protein and p97 (3, 4). The recently solved crystal structure of the first nucleotide binding domain of ClpB also demonstrated the close structural relationship between Clp/Hsp100 and AAA proteins (5). The conserved AAA-domain (also referred to as AAA module) is made up of two domains, a core region that forms the nucleotide binding pocket, containing the classical Walker A and B motifs, and a C-terminal α-helical domain (C-domain). The ATP binding pocket is located at the interface of neighboring subunits in the oligomer. The C-domain contacts its own core ATPase domain and that of adjacent subunits and is involved in nucleotide binding and hexamerization in HsIu (3, 4). Besides sensing the nucleotide status of the core ATPase domain, C-terminal domains of the second AAA-domain have also been proposed to mediate substrate interaction and were therefore termed the sensor and substrate discrimination (SSD)2 domains (6).

In addition Hsp100/Clp proteins contain variable regions at their N terminus. ClpA and ClpB have homologous N-domains of about 150 residues that consist of two sequence repeats and form an independent structural domain still of unknown function (7). ClpX possesses a zinc binding domain at the N terminus (8), whereas HsIu lacks an N-terminal domain but rather contains an extra domain (the I-domain) inserted into the AAA-domain (3, 4). The most striking difference between members within the class I subfamily is the presence/absence of a region that is proposed to link the two AAA-domains ATP-1 and ATP-2. The presence of this variable linker region serves as a criteria for classification of Hsp100 proteins; the linker is longest in ClpB (~140 residues) but is absent in ClpA (see Fig. 1) (1).

ClpB is unique among the Hsp100/Clp proteins because it does not associate with a proteolytic partner protein. Recently, an essential docking site of the peptidase ClpP was identified within ClpX (9). The signature motif (LIV-G-F-L) is conserved in all other ClpP-interacting proteins like ClpA but is missing in ClpB, thereby explaining why ClpB acts independently of peptidases. Instead ClpB mediates the resolubilization of aggregated proteins in cooperation with the DnaK chaperone system (10–13). The mechanism of the disaggregation reaction and the basis of ClpB/DnaK cooperation are still not understood.

Here we report a structure-function analysis of ClpB that is

* This work was supported by Deutsche Forschungsgemeinschaft Grant Bu617/14-1 and by the Fond der Chemischen Industrie (to B. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence may be addressed. E-mail: a.mogk@zmbh.uni-heidelberg.de.
‡ To whom correspondence may be addressed. E-mail: bukau@zmbh.uni-heidelberg.de.
§ Institut für Biochemie und Molekularbiologie, Universität Freiburg, Hermann-Herder-Strasse 7,
79104 Freiburg, Germany
aimed at identifying the roles of individual domains and conserved motifs in the disaggregation process and the coupling of the ATPase cycle with the chaperone activity. Constructed ClpB variants were characterized with respect to their structural integrity, as determined by oligomerization studies and partial proteolysis. Additionally the ATPase and chaperone activities of all constructs were tested.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—E. coli strains used were derivatives of MC4100 (araD139 ΔargF-lacU169 rpsL150 relA1 fliB3501 deoC1 ptsF25 rbs). clpB mutant allelic was introduced by P1 transduction into the ΔclpB strain to generate ΔclpB null. The E. coli clpB gene was cloned by PCR into the pUHE21 expression vector and verified by DNA sequencing. Mutants and deletion variants were generated by using standard PCR techniques. ClpB-(410–532) was constructed by replacing the entire linker region with an SpeI site, leading to the insertion of two amino acids (Thr and Ser) at the deletion site. Reinsertion of the linker was obtained by PCR amplification of the corresponding region (amino acid 410–530) and addition of an SpeI site 5’ and an NheI site 3’ to the linker fragment. This fragment was digested with NheI/SpeI and inserted into the SpeI site of the Δ410–532 construct, leading to the insertion of two amino acids (Thr and Ser or Ala and Ser) at each domain boundary (ClpB Δ ΔL). Each mutagenesis was confirmed by DNA sequencing.

**Proteins**—Wild type and mutant ClpB were purified as described after overproduction in ΔclpB::kan cells (11). Purifications of DnaK, DnaJ, and GrpE were according to published protocols (14). Pyruvate kinase and α-casein were purchased from Sigma; malate dehydrogenase (MDH) was from pig heart muscle, and firefly luciferase from Roche Applied Science. Protein concentrations were determined with the BioRad Bradford assay using bovine serum albumin as standard. Protein concentrations refer to the proteomer.

**Tryptophan Fluorescence**—Measurements of the intrinsic tryptophan fluorescence of ClpB were performed on a PerkinElmer Life Sciences LS50B spectrofluorimeter. The emission spectra of tryptophan fluorescence of ClpB (0.5 μM) in the absence of nucleotide or the presence of 2 mM ATP/ADP were recorded at 30 °C in buffer A (50 mM Tris, pH 7.5, 150 mM KCl, 20 mM MgCl2, 2 mM DTT) containing 0.5 μM ClpB (wild type or derivatives), 2 mM ATP, and [α-32P]ATP (0.1 μCi, Amersham Biosciences). ATPase activities were also determined in the presence of 0.25 mg/ml α-casein or 100 mM ammonium sulfate. Hydrolysis was quantified by using the program MACBAS version 2.5 (Fuji), and rates of ATP hydrolysis were determined from the linear increase of luciferase activities between 15 and 30 min.

**ATPase Activity Assay**—ATP hydrolysis rates under steady-state conditions were as described (14). Reactions were performed at 30 °C in buffer A (50 mM Tris, pH 7.5, 150 mM KCl, 20 mM MgCl2, 2 mM DTT) containing 0.5 μM ClpB (wild type or derivatives), 2 mM ATP, and [α-32P]ATP (0.1 μCi, Amersham Biosciences). ATPase activities were also determined in the presence of 0.25 mg/ml α-casein or 100 mM ammonium sulfate. Hydrolysis was quantified by using the program MACBAS version 2.5 (Fuji), and rates of ATP hydrolysis were determined from the linear increase of luciferase activities between 15 and 30 min.

**Size Exclusion Chromatography**—Size exclusion chromatography was performed at room temperature in buffer A containing 5% (v/v) glycerol. Nucleotide-dependent oligomerization was followed in the presence of 2 mM ATP or ADP in the running buffer. 10 μM ClpB was incubated in buffer A in the absence or presence of nucleotides (2 mM ATP or ADP) for 5 min at room temperature, followed by injection into the high pressure liquid chromatography system (PerkinElmer Life Sciences) connected to a SEC 400 column (Bio-Rad). Chromatographic steps were performed with a flow rate of 0.8 ml/min.

**Cross-linking Experiments**—All ClpB variants were disulfide against buffer B (50 mM Hepes, pH 7.5, 50 mM KCl, 20 mM MgCl2, 2 mM DTT). 1 μM ClpB was incubated at 30 °C in the absence or presence of nucleotides (2 mM ATP or ADP) for 5 min. Cross-linking reactions were started by addition of 0.1% glutaraldehyde and incubated for another 10 min. Reactions were stopped by addition of 1 μM Tris, pH 7.5, and cross-linking products were analyzed by SDS-PAGE (4–15%) followed by silver staining.

**Partial Proteolysis and Identification of Cleavage Products**—1 μM ClpB (wild type or derivative) was incubated at 30 °C in buffer A without DTT for 5 min in the absence or presence of nucleotide (2 mM ATP, ADP, and ATPS). Proteolysis was initiated upon addition of 0.2 μg/ml thermolysin or subtilisin, and generated cleavage products were analyzed on SDS-PAGE (15%) and silver staining. Kinetic analysis of the degradation reaction revealed the occurrence of stable fragments after 30–60 min of incubation time. Identity of cleavage products was determined by N-terminal sequencing (TopLab) and mass spectrometry. For mass spectrometry analysis, bands were excised from one-dimensional Coomassie Silver-stained SDS-polyacrylamide gels and in-gel digested with trypsin as described (15). Tryptic peptides were analyzed by nanoelectrospray tandem mass spectrometry as described previously (16) using a QSTAR™ Pulsar (MDS Sciex, Toronto, Canada) equipped with a nanoelectrospray ion source (MDS Proteomics, Odense, Denmark). Sequence searches were performed with the Protein and Peptide Software Suite (MDS Proteomics).

**In Vitro Activity Assays**—2 μM MDH was denatured in buffer A for 30 min at 47 °C. Turbidity of 1 μM aggregated MDH was measured at 30 °C at an excitation and emission wavelength of 550 nm (PerkinElmer Life Sciences luminescence spectrometer LS50B). Decrease of light scattering was followed upon addition of 1.5 μM ClpB (wild type or derivative), the DnaK chaperone system (1 μM DnaK, 0.2 μM DnaJ, 0.05 μM GrpE), and an ATP-regenerating system (3 mM phosphoenolpyruvate, 20 ng/ml pyruvate kinase, 2 mM ATP). Disaggregation rates were derived from the linear decrease of turbidity between 5 and 30 min.

**RESULTS**

**Design of ClpB Mutants and Deletions Variants**—ClpB is proposed to consist of an N-terminal domain, two AAA-domains (ATP-1 and ATP-2), which are separated by a linker region, and the C-terminal SSD domain (as illustrated in Fig. 1). In order to probe the proposed domain organization of ClpB, several deletion variants were constructed, based on sequence and secondary structure analysis of ClpB and its comparison with AAA+ proteins of known structure. Due to the presence of an internal start codon, two versions of E. coli ClpB exist in vivo: a full-length protein (aa 1–857) and an N-terminally truncated variant (aa 149–857). Both versions have been demonstrated to form mixed oligomers (18). In order to work with uniform proteins, we decided to mutate the internal start codon of wild type ClpB, leading to the production of full-length ClpB only. This ClpB derivative exhibited in vitro and in vivo chaperone activity, indistinguishable from ClpB expressed from the wild type gene (data not shown). The corresponding construct was used as basis for the construction of all ClpB derivatives.

The border between the N-domain and the first AAA-domain is often characterized by the presence of an internal start codon within the clpB gene (Val149). Sequence analysis of Hsp100 N-domains and secondary structure prediction of 15 different ClpB proteins revealed that the ClpB N-domain is built up of residues 1–144 (7) (data not shown). We therefore decided to use Met-143 as the start site for ClpB clpB AN (143–857) version. The C-terminal SSD was removed in the ClpB ASSD variant (aa 1–758). AAA-domains were suggested to be separated by the insertion of the linker region within ClpB. Recent sequence and structural analysis of AAA+ proteins revealed that the linker region potentially interrupts the first AAA-domain instead of separating both AAA modules (2). Several ClpB deletion variants were constructed to test this possibility: ClpB-(1–409), ClpB-(551–857), and ClpB-(1–567).

Several conserved motifs and residues are proposed to be involved in ATP binding and hydrolysis by AAA proteins. The highly conserved Lys residues of the Walker A motif (Lys-212...
and Lys-611 of ClpB) contact the phosphate groups of the β and γ nucleotides in the first and second AAA-domain, respectively (19). The conserved Glu residues of the Walker B motif (Glu-279 and Glu-678 of ClpB) are proposed to represent the catalytic base for ATP hydrolysis in the first and second AAA-domain, respectively. AAA+ proteins additionally contain a conserved Arg residue, termed sensor 2 (2). Sensor 2 lies in the C-terminal domain of each AAA module and contacts the γ-phosphate of bound ATP. This Arg residue is part of an invariant GAR motif (813GAR815 in ClpB) within the SSD domain of AAA+ (Hsp100) proteins. It is proposed that the conserved arginine can sense the nucleotide status and mediate conformational changes of the C-terminal domain relative to the core domain during ATP hydrolysis (19, 20).

The formation of hexameric rings in AAA+ proteins brings residues of adjacent protomers into close proximity to ATP, bound to a neighboring subunit. Such interactions could provide the basis for an intermolecular catalytic mechanism, resulting in cooperative ATP hydrolysis within the oligomer. Previous studies on the AAA protein FtsH have demonstrated tree that isolated AAA-domains (1–409 and 551–857) stayed as monomers in presence of glutaraldehyde, whereas in case of the ClpB SSD variant (aa 1–758) and a longer version of the first AAA module (aa 1–567) some dimeric and trimeric species were observed (Fig. 3B). Mutating the Walker A motif (K212A) or the Arg finger (R332A) of the first AAA-domain resulted in the formation of mixtures of oligomeric species, ranging from monomers to tetramers in case of K212A and monomers to hexamers for R332A (Fig. 3A and Table I). Interestingly, the observed oligomerization defects were nucleotide-independent, indicating that the mutated residues are also important for subunit interactions within the oligomer in the absence of ATP. The involvement of these charged residues in ClpB assembly can also explain the observed salt sensitivity of ClpB and Hsp104 oligomerization (24, 25). All other ClpB variants exhibited cross-linking characteristics indistinguishable from wild type ClpB with exception of ClpB-(Δ410–532) that misses the linker region. This variant exhibited only a ladder of cross-linking products in the absence of nucleotide and required ATP for full oligomerization. The observed assembly defect was, however, not primarily caused by the absence of the linker region but rather by the introduction of additional amino acids at each boundary of the linker segment, resulting from the construction strategy of this deletion variant (see “Experimental Procedures”). Thus a control construct (termed ClpB ΔLBL) carrying the same additional amino acids and the reinserted linker region, also exhibited the same oligomerization defects as ClpB-(Δ410–532) (data

In an additional approach, oligomerization characteristics were studied by glutaraldehyde cross-linking. Fast cross-linking of ClpB monomers to oligomeric species was obtained within 10 min in the absence of nucleotide, indicating that ClpB assembly can in principle also occur without nucleotide. Because such nucleotide-independent hexamerization was not observed during gel filtration runs, the formed oligomers seem to be unstable in the absence of ATP.

Kinetic analysis of the cross-linking reaction revealed that a ladder of cross-linking products preceded the formation of the fully cross-linked oligomer (data not shown). The presence of ATP or ADP accelerated the cross-linking reaction and also slightly changed the size of the fully cross-linked oligomer. Whereas the ladder of cross-link products could be followed up to a heptamer in the absence of nucleotide, addition of ATP and ADP resulted predominantly in cross-linking to the hexameric and pentameric species, respectively (Fig. 3A). The existence of heptameric ClpB rings have also been shown by Chung and co-workers (18) using electron microscopy.

Cross-linking studies of ClpB mutants and deletion variants confirmed the findings obtained by gel filtration analysis; however, qualitative differences with respect to the oligomerization deficiencies were revealed. Isolated AAA-domains (1–409 and 551–857) stayed as monomers in presence of glutaraldehyde, whereas in case of the ClpB ΔSSD variant (aa 1–758) and a longer version of the first AAA module (aa 1–567) some dimeric and trimeric species were observed (Fig. 3B). Mutating the Walker A motif (K212A) or the Arg finger (R332A) of the first AAA-domain resulted in the formation of mixtures of oligomeric species, ranging from monomers to tetramers in case of K212A and monomers to hexamers for R332A (Fig. 3A and Table I). Interestingly, the observed oligomerization defects were nucleotide-independent, indicating that the mutated residues are also important for subunit interactions within the oligomer in the absence of ATP. The involvement of these charged residues in ClpB assembly can also explain the observed salt sensitivity of ClpB and Hsp104 oligomerization (24, 25). All other ClpB variants exhibited cross-linking characteristics indistinguishable from wild type ClpB with exception of ClpB-(Δ410–532) that misses the linker region. This variant exhibited only a ladder of cross-linking products in the absence of nucleotide and required ATP for full oligomerization. The observed assembly defect was, however, not primarily caused by the absence of the linker region but rather by the introduction of additional amino acids at each boundary of the linker segment, resulting from the construction strategy of this deletion variant (see “Experimental Procedures”). Thus a control construct (termed ClpB ΔLBL) carrying the same additional amino acids and the reinserted linker region, also exhibited the same oligomerization defects as ClpB-(Δ410–532) (data
The presence of nucleotides (2 mM ATP/ADP) in the running buffer (50 mM WT/H11002) were recorded in the absence (tives analyzed by gel filtration chromatography. A (25, 26). Consistently, the ATP-dependent blue shift was re-

ties were obtained (Fig. 5 A). The larger degradation products (aa 3–331 and 3–857) corresponded now to the complete core domain of the first AAA module. The second fragment (aa 536–756) represented the core domain of the second AAA module. Addition of ATP or ADP slowed the proteolysis considerably and resulted in stabilization of full-length ClpB and the occurrence of two other stable cleavage products. The first fragment (aa 3–331 and 3–351) was already obtained by cleavage in the absence of nucleotide, although the second fragment (aa 537–857 and 551–857) corresponded now to the complete second AAA module. In the presence of ATP/γS cleavage was further limited, and reduced amounts of the smaller fragments were obtained (Fig. 5A).

Partial proteolysis of ClpB variants revealed that nucleotide-dependent stabilization of wild type ClpB can be attributed to hexamer formation. Mutants with oligomerization deficiencies (K212A, ClpB ASSD) or reduced stability of hexamers (ClpB-(410–532)) were degraded more quickly in the presence of ATP compared with WT ClpB and thus did not exhibit stabilization of the full-length version (Fig. 5C). The observed defects of ClpB-(410–532), missing the linker region, in ATP-dependent stabilization could again be attributed to the presence of the two additional amino acids at the domain boundary because the control construct (ClpB ΔLβLβ) with the reinserted linker region exhibited the same reduced proteolytic stability. Isolated AAA-domains (1–409 and 551–857) did not react to ATP addition and were processed rapidly to stable cleavage products (similar to the fragments obtained for WT ClpB in the

![Diagram](https://example.com/diagram.png)

**Fig. 2.** Oligomerization of ClpB wild type and mutant derivatives analyzed by gel filtration chromatography. A, elution profiles of ClpB wild type (WT) were recorded in the absence (-) or presence of nucleotides (2 mM ATP/ADP) in the running buffer (50 mM Tris, pH 7.5, 20 mM MgCl₂, 150 mM KCl, 10% (v/v) glycerol). Elution positions of protein standards (thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and IgG (158 kDa)) are given. B, elution profiles of the indicated ClpB mutants were recorded in the presence of ATP (2 mM) in the running buffer.

not shown), showing that the linker is not essential for oligomerization.

Finally in a complementary approach we examined the intrinsic fluorescence of ClpB as a potential tool to monitor the oligomeric state. ClpB contains 2 tryptophan residues (Trp-462 and Trp-543). We determined whether tryptophan fluorescence changes in a nucleotide-dependent manner. In the absence of nucleotide, ClpB exhibited a fluorescence emission maximum at 350.5 nm. Addition of ATP or ADP caused a blue shift in the fluorescence maximum to 346.5 nm, and a stronger decline of fluorescence intensity beyond the maximum was observed (Fig. 4A). This change was more pronounced in the presence of ATP compared with ADP. Several findings suggest that the observed changes in fluorescence are caused by oligomerization of ClpB. First, the blue shift was also observed when ClpB was incubated in low salt buffer in the absence of nucleotide (Fig. 4). Such buffer conditions have been shown for several ClpB homologues to stabilize the oligomer in the absence of nucleotide (25, 26). Consistently, the ATP-dependent blue shift was re-vered under high ionic strength conditions (addition of 100 mM (NH₄)₂SO₄ or 300 mM NH₄Cl), conditions which have been shown to inhibit ClpB activity (10). De-oligomerization of ClpB in the additional presence of 100 mM (NH₄)₂SO₄ or 300 mM NH₄Cl was also observed in cross-linking experiments. Such high salt conditions resulted in a strong reduction of ClpB cross-linking efficiency in the absence or presence of ATP (Fig. 4B and data not shown).

Second, the observed changes in fluorescence also became nucleotide-independent with increasing ClpB concentrations; a complete blue-shift was revealed in presence of 4 µM ClpB (data not shown). Finally, the analysis of ClpB mutants and deletion variants revealed a direct correlation between the ability to form hexamers and the observed changes in tryptophan fluorescence; the ATP-dependent blue shift was not or was only partially observed for ClpB variants with oligomerization defects (Table I).

In order to dissect the individual contributions of the two tryptophan residues to the changes in the fluorescence spectrum, a ClpB variant (W543F) with only a single tryptophan residue (Trp-462) was constructed. Although this variant was not affected in oligomerization and exhibited full chaperone activity in vitro, nucleotide-dependent changes in ClpB fluorescence were no longer observed (data not shown). We conclude that conformational changes in the close vicinity of Trp-543, driven by oligomerization, must be responsible for the observed blue shift in tryptophan fluorescence of ClpB.

**Nucleotide-dependent Conformational Changes within ClpB Revealed by Partial Proteolysis—**The ability of Hsp100 mutants to form oligomers is commonly used as a criteria for their structural integrity. We additionally looked for conformational changes of wild type ClpB in response to nucleotides, and we checked whether such structural rearrangements were preserved in the constructed ClpB variants. ClpB was subjected to limited proteolysis by thermolysin or subtilisin in the absence or presence of nucleotides. In the absence of nucleotides two highly stable fragments were recovered (Fig. 5A). Fragments were N-terminally sequenced and identified by mass spectrometry (Fig. 5B). The larger degradation products (aa 3–331 and 3–351) corresponded to the N-terminal domain of ClpB and the core domain of the first AAA module. The second fragment (aa 536–756) represented the core domain of the second AAA module. Addition of ATP or ADP slowed the proteolysis considerably and resulted in stabilization of full-length ClpB and the occurrence of two other stable cleavage products. The first fragment (aa 3–331 and 3–351) was already obtained by cleavage in the absence of nucleotide, although the second fragment (aa 537–857 and 551–857) corresponded now to the complete second AAA module. In the presence of ATP/γS cleavage was further limited, and reduced amounts of the smaller fragments were obtained (Fig. 5A).
absence of nucleotides). In contrast, a longer version of the first AAA-domain (1–567) in the presence of ATP exhibited some protection from degradation. Further structural analysis of ClpB point mutants surprisingly revealed that conformational changes within each AAA-domain can occur independently of structural deficiencies in the other AAA module. Thus, the Walker A mutant K212A of the first AAA-domain, although deficient in oligomerization, still exhibited ATP-dependent conformational changes in the second AAA-domain (stabilization of the second AAA module). On the other hand structural changes within the second AAA-domain were not or were only partially observed in the case of K611A and 813AAA815, ClpB mutants that nevertheless showed stabilization of the full-length protein (Fig. 5C). Thus oligomerization protects full-length ClpB even in case of mutants that do not bind nucleotide tightly at the second AAA module (K611A; 813AAA815). We conclude that stabilization of full-length ClpB against proteolytic degradation primarily reflects binding of ATP to the first AAA-domain. These data also demonstrate that the ability to form oligomers is not per se a sufficient criteria for probing the structural integrity of the ClpB mutants. Structural integrity with respect to oligomerization and stability during partial proteolysis was only completely preserved in Walker B mutants of ClpB, although Walker A and the sensor 2 mutants exhibited significant structural deficiencies. Interestingly, the double Walker B mutant (E279A/E678A) was much more re-

### Table I

**Oligomerization of ClpB fragments and mutants**

Assembly of the indicated ClpB derivatives was analyzed in the presence of 2 mM ATP by gel filtration chromatography, cross-linking, and tryptophan fluorescence. Size of ClpB oligomers were calculated from a calibration curve with protein standards. Cross-link products formed after 10 min of incubation with glutaraldehyde were analyzed by SDS 4–12% PAGE. Most populated species are underlined. ATP-dependent oligomerization of ClpB is coupled to a blue shift in the maximum of fluorescence (346.5 nm instead of 350.5 nm in the absence of nucleotide). The maximum of ClpB fluorescence is indicated. ND, not determined.

<table>
<thead>
<tr>
<th>Gel filtration chromatography</th>
<th>Cross-linking products</th>
<th>Maximum of ClpB fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ClpB fragments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–857 (wild type)</td>
<td>Hexamer</td>
<td>Hexamers</td>
</tr>
<tr>
<td>143–857 (∆N)</td>
<td>Hexamer</td>
<td>Hexamers</td>
</tr>
<tr>
<td>1–758 (∆SSD)</td>
<td>Monomer</td>
<td>Monomers-trimers</td>
</tr>
<tr>
<td>∆410–532 (∆linker)</td>
<td>Hexamer</td>
<td>Hexamers</td>
</tr>
<tr>
<td>1–409</td>
<td>Monomer</td>
<td>Monomer</td>
</tr>
<tr>
<td>1–567</td>
<td>Monomer</td>
<td>Monomer</td>
</tr>
<tr>
<td>551–857</td>
<td>Monomer</td>
<td>Monomers-dimers</td>
</tr>
<tr>
<td>ClpB mutants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K212A (Walker A)</td>
<td>Dimer</td>
<td>Monomers-tetramers</td>
</tr>
<tr>
<td>K611A (Walker A)</td>
<td>Hexamer</td>
<td>Hexamers</td>
</tr>
<tr>
<td>K212A/K611A</td>
<td>Dimer</td>
<td>Monomers-tetramers</td>
</tr>
<tr>
<td>E279A (Walker B)</td>
<td>Hexamer</td>
<td>Hexamers</td>
</tr>
<tr>
<td>E678A (Walker B)</td>
<td>Hexamer</td>
<td>Hexamers</td>
</tr>
<tr>
<td>E279A/E678A</td>
<td>Hexamer</td>
<td>Hexamers</td>
</tr>
<tr>
<td>R532A (Arg finger)</td>
<td>Dimer</td>
<td>Monomer-hexamers</td>
</tr>
<tr>
<td>R756A (Arg finger)</td>
<td>Hexamer</td>
<td>Hexamers</td>
</tr>
<tr>
<td>813AAA815 (sensor 2)</td>
<td>Hexamer</td>
<td>Hexamers</td>
</tr>
</tbody>
</table>

**Fig. 3.** Assembly of ClpB wild type and derivatives revealed by cross-linking. A, 1 μM ClpB wild type (WT) and the indicated point mutants were incubated for 5 min at 30 °C in the absence of nucleotides (lane 2) or the presence of 2 mM ATP (lane 3) or ADP (lane 4). Cross-linking reactions were initiated by addition of glutaraldehyde and proceeded for 10 min. Cross-linked proteins were separated on SDS (4–12%)-polyacrylamide gels, followed by silver staining. Incubation of ClpB proteins in the absence of cross-linker (lane 1) served as control. B, ClpB deletion variants were incubated in the absence (lane 2) or presence of 2 mM ATP (lane 3) for 5 min at 30 °C. Cross-linking was performed as described above. Incubation of truncated ClpB species in the absence of cross-linker (lane 1) served as control.
sistant toward proteolysis than the single Walker B mutants and ClpB wild type. Because this mutant is deficient in ATP hydrolysis (see below), the occurrence of the other fragments (aa 3–331 and 3–351 and 537–857 and 551–857) in ClpB wild type (and various ClpB mutants) can be attributed to ATP hydrolysis occurring during the digestion reaction. These data also indicate that ClpB therefore adopts a different conformation if both AAA-domains have ATP bound, as compared with the situation where only one AAA-domain has ATP bound and the other domain has ADP bound.

**ATPase Activities of ClpB Mutants and Deletion Variants**

ATPase activities of ClpB variants were determined in the absence or presence of the artificial substrate casein, which is known to stimulate ATP hydrolysis by ClpB (27). Wild type ClpB exhibited an ATPase rate of 0.021/s, and the ATPase activity was increased by 3–4-fold in the presence of saturating concentrations of α-casein (20-fold excess over ClpB monomers). ClpB ΔN had a slightly increased ATPase activity but was less stimulable by casein, in agreement with published data (23, 28). Removal of the linker region (ClpB-(Δ410–532)) strongly decreased the basal ATPase activity by 4-fold, although stimulation by casein was not affected. Similar results were obtained with the control construct ClpB ΔLBL, bearing a reinserted linker, indicating that the reduced basal ATPase activity is primarily caused by the observed oligomerization deficiencies. All other deletion variants (aa 1–409, 1–567, 551–857, and 1–758) with even stronger defects in oligomerization did not exhibit any ATPase activity, even in presence of casein, indicating that ATP binding and/or hydrolysis is strictly linked to the formation of hexamers. Consistent with this hypothesis, the basal ATPase activities of mutants with oligomerization defects were sensitive to the buffer conditions; ATP activities of K212A and Arg-332 were enhanced 2–3-fold in low salt buffer, conditions that favor oligomerization (data not shown). In agreement with these findings, addition of 100 mM (NH₄)₂SO₄ or 300 mM NH₄Cl diminished the ATPase rate of full-length ClpB or ClpB ΔN 3- and 5-fold, respectively (Table II, data not shown). Further analysis of Walker A and Walker B single mutants, bearing only one functional AAA-domain, revealed that especially the first AAA-domain was sensitive toward high

**FIG. 4.** ATPase Activities of ClpB Mutants and Deletion Variants—

ATPase activities of ClpB mutants were determined in the presence of nucleotide or in the presence of 2 mM ATP/ADP/ATP·S for 60 min. Cleavage products were separated on SDS (15%)-polyacrylamide gels, followed by silver staining.

**FIG. 5.** Nucleotide-dependent structural changes in ClpB revealed by limited proteolysis. A, partial proteolysis of ClpB wild type by thermolysin and subtilisin was performed in the absence of nucleotide or in the presence of 2 mM ATP/ADP/ATP·S for 60 min. Cleavage products were separated on SDS (15%)-polyacrylamide gels, followed by silver staining.
ionic strength conditions. Addition of 100 mM (NH₄)₂SO₄ strongly reduced the ATPase activity of mutants, in which the second AAA-domain was inactivated (K611A and E678A), whereas variants with only an active second AAA module (E279A and K211A) were partially affected (Table II), thereby underlining the functional importance of the first AAA-domain for ClpB assembly. Higher concentrations of ammonium sulfate (200 mM) completely inhibited ATP hydrolysis by ClpB (data not shown).

Single Walker A or Walker B mutations resulted in a complete loss of ATPase activity in the corresponding AAA-domain, because double Walker A or Walker B mutants were deficient in ATP hydrolysis, even in presence of casein. Because significant ATPase activities were measurable for all Walker A and Walker B single mutants, both ATPase domains seemed to contribute to the basal ATPase activity of ClpB. Additionally, both AAA modules were stimulated to similar degrees by casein. Variations in the basal ATPase activities of ClpB point mutants compared with wild type ClpB indicate a communication between both AAA-domains. The basal ATPase rate of ClpB E678A was increased 4-fold compared with wild type. Additionally, mutations in the linker region (ClpB-LBL with the SSD motif) also caused a complete loss of the ATPase activity of ClpB. Importantly, this inactivation was not caused by the observed oligomerization defects of ClpB—casein (0.25 mg/ml). Besides stabilization of the oligomeric state, conformational changes within ClpB, triggered by substrate binding, may also contribute to ATP hydrolysis, because saturating ClpB concentrations (1 μM) still showed a lower specific ATPase activity in the absence of casein (Fig. 6).

**Structure-Function Analysis of ClpB**

ATPase activities of the indicated ClpB derivatives were determined in buffer A. The factors of ATPase stimulation in the presence of 0.25 mg/ml α-casein and the factors of ATPase inhibition in the presence of 100 mM ammonium sulfate are given. ND, not determined.

<table>
<thead>
<tr>
<th>ClpB fragments</th>
<th>ATPase activity (μs⁻¹)</th>
<th>Factor of ATPase stimulation by α-casein</th>
<th>Factor of ATPase inhibition by sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–857 (wild type)</td>
<td>0.021</td>
<td>3.3</td>
<td>3</td>
</tr>
<tr>
<td>143–857 (ΔN)</td>
<td>0.037</td>
<td>1.9</td>
<td>4.9</td>
</tr>
<tr>
<td>1–788 (ΔSSD)</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Δ410–532 (linker)</td>
<td>0.005</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>1–409</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>1–567</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>E678A</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>551–857</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>ClpB mutants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K212A (Walker A)</td>
<td>0.006</td>
<td>4.4</td>
<td>1.4</td>
</tr>
<tr>
<td>K611A (Walker A)</td>
<td>0.019</td>
<td>5.3</td>
<td>4.2</td>
</tr>
<tr>
<td>K212A/K611A</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>E278A (Walker B)</td>
<td>0.023</td>
<td>3.8</td>
<td>1.7</td>
</tr>
<tr>
<td>E678A (Walker B)</td>
<td>0.039</td>
<td>6.6</td>
<td>1.7</td>
</tr>
<tr>
<td>E279A/E678A</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>R332A (Arg finger)</td>
<td>0.019</td>
<td>2.4</td>
<td>ND</td>
</tr>
<tr>
<td>R756A (Arg finger)</td>
<td>0.003</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Ψ15AAAS15 (SSD motif)</td>
<td>0.026</td>
<td>2.8</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Table II**

ATPase activities of ClpB fragments and mutants

In contrast, in the presence of casein high ATPase activities were already determined at low ClpB concentrations (0.1 μM), implying that stabilization of ClpB hexamers by substrates causes induction of the ATPase activity. Such stabilization by casein was indeed observed by gel filtration analysis for the ClpB Walker B mutant E279A/E678A, which is deficient in ATP hydrolysis. Besides stabilization of the oligomeric state, conformational changes within ClpB, triggered by substrate binding, may also contribute to the stimulation of ATP hydrolysis, because saturating ClpB concentrations (1 μM) still showed a lower specific ATPase activity in the absence of casein (Fig. 6).

**Chaperone Activities of ClpB Derivatives**—We tested the chaperone activities of the various ClpB variants in vitro by following the ClpB/DnaK-dependent reactivation of heat-aggregated MDH and firefly luciferase. Resolubilization of MDH aggregates by ClpB/DnaK was directly followed by measuring the decrease of aggregate turbidity in light scattering experiments. Disaggregation of aggregated luciferase was followed by determining the refolding rate of luciferase in the presence of the bi-chaperone system. All disaggregation reactions were performed in the presence of non-saturating ClpB concentrations which allowed for sensitive detection of potential activity defects. Results are summarized in Table III.

All ClpB deletion variants (an 1–409, 1–567, 551–857, and 1–758) with severe oligomerization defects were inactive in MDH and luciferase disaggregation. Deletion of the linker region (ClpB-(Δ410–532)) also caused a complete loss of the disaggregation activity of ClpB. Importantly, this inactivation was not caused by the observed oligomerization deficiency of ClpB-(Δ410–532), because the control construct ClpB LBL with the reinserted linker region exhibited significant disaggregation activity (60% of ClpB wild type; data not shown). ClpB ΔN had the same chaperone activity as full-length ClpB. Full disaggregation activity of ClpB ΔN was also observed when the ClpB concentrations in the activity assays were further reduced, thereby increasing the dependence of the disaggregation on ClpB (data not shown).

ClpB variants with point mutations in conserved motifs exhibited none or only partial chaperone activity (Table III). In general a disaggregation activity was only observed for mutants that are still able to form oligomers. Thus the Walker A (K212A) and Arg finger (R332A) mutations of the first AAA-domain, which are deficient in hexamer formation, were inac-

---

2 J. Weibezahn, C. Schlieker, B. Bukau, and A. Mogk, manuscript in preparation.
TABLE III

Structure-Function Analysis of ClpB

Chaperone activities of ClpB mutants and fragments

Chaperone activity (disaggregation rate of aggregated MDH; refolding rate of aggregated luciferase) of ClpB wild type was set as 100%. In vivo activity reflects the ability of ClpB derivatives to restore thermostability and protein disaggregation in E. coli ΔclpB mutant cells (+, full activity; −, residual activity; nd, no activity). ND, not determined.

<table>
<thead>
<tr>
<th>ClpB fragments</th>
<th>Disaggregation of aggregated MDH</th>
<th>Refolding of aggregated luciferase</th>
<th>In vivo activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ClpB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–857 (野生 type)</td>
<td>100</td>
<td>100</td>
<td>++</td>
</tr>
<tr>
<td>143–857 (ΔN)</td>
<td>98</td>
<td>99</td>
<td>+</td>
</tr>
<tr>
<td>1–758 (ΔSSD)</td>
<td>0</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>Δ340–551 (Viewer)</td>
<td>0</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>1–409</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>1–567</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>551–857</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>ClpB mutants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K212A (Walker A)</td>
<td>0</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>K611A (Walker A)</td>
<td>4–5</td>
<td>8–10</td>
<td>(−)</td>
</tr>
<tr>
<td>K212A/K611A</td>
<td>0</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>E279A (Walker B)</td>
<td>7–9</td>
<td>8–10</td>
<td>(−)</td>
</tr>
<tr>
<td>E678A/Walker B</td>
<td>2–3</td>
<td>4–5</td>
<td>(−)</td>
</tr>
<tr>
<td>E279A/E678A</td>
<td>0</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>R332A (Arg finger)</td>
<td>0</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>R756A (Arg finger)</td>
<td>0.5–1</td>
<td>0.5–1</td>
<td>−</td>
</tr>
<tr>
<td>813AA815 (SSD motif)</td>
<td>0</td>
<td>0</td>
<td>−</td>
</tr>
</tbody>
</table>

Fig. 7. In vivo activity of ClpB derivatives. A, E. coli wild type (MC1400), ΔclpB mutant cells, and ΔclpB mutants, bearing plasmid-encoded clpB alleles under the control of an IPTG-regulatable promoter, were grown in LB medium at 30 °C in the presence of 25 μM IPTG to mid-exponential phase. Subsequently strains were heat-shocked to 50 °C and incubated for the indicated time. Various dilutions of the stressed cells were spotted on LB plates and incubated at 30 °C. After 24 h, colony numbers were counted, and survival rates were calculated in relation to unstressed cells. B, cultures of ΔclpB mutant strains, bearing plasmid-encoded clpB alleles under the control of an IPTG-regulatable promoter, were grown in LB medium in the presence of 25 μM IPTG at 30 °C to logarithmic phase. Cells were then shifted to 45 °C for 30 min, followed by a recovery phase at 30 °C for 60 min. Aggregated proteins were isolated before (0 min) and after the recovery phase (60 min) and analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue.


discussive. In contrast, the corresponding mutations in the second AAA-domain (K611A and R756A) exhibited low chaperone activities to varying degrees (below 10% of the WT control). Partial activities were also obtained in case of single Walker B mutants, although the double mutant (E279A/E678A) did not have any disaggregation activity. These findings indicate that whereas ATP hydrolysis is strictly required for chaperone activity, a lack of ATP hydrolysis in one AAA-domain does not completely inhibit the disaggregation activity.

Disaggregation activities of all ClpB variants were also tested in vivo by complementation of the phenotypes of ΔclpB mutant cells by IPTG induction of plasmid-encoded clpB mutant alleles. Resolubilization of protein aggregates, formed during severe heat stress to 45 °C in E. coli cells, is severely affected in ΔclpB mutants. This deficiency is directly linked to a strongly reduced survival rate of ΔclpB mutant cells at lethal (50 °C) temperatures compared with wild type cells (thermotolerance). We therefore tested the ClpB variants for their ability to re-establish protein disaggregation and thermostolerance in ΔclpB mutant cells (Fig. 7). Complementation studies were performed in the presence of 25 μM IPTG, leading to 3–4-fold increased ClpB levels as compared with heat-shocked wild type cells lacking any plasmid (data not shown). In summary the in vivo disaggregation activities reflected those observed in vitro. Only wild type ClpB and the ClpB ΔN could efficiently mediate the resolubilization of protein aggregates and the development of thermostolerance (Fig. 7 and Table III). Constructs with partial chaperone activities in vitro, such as E279A, also exhibited some activity in vivo.

DISCUSSION

Here we present a detailed structure-function analysis of the AAA+ chaperone ClpB, which mediates resolubilization of protein aggregates in cooperation with the DnaK chaperone system. Oligomerization of ClpB was dependent on both AAA-domains; however, each domain seemed to play a different role in ClpB assembly. Deletion of the α-helical C-domain of the second AAA-domain (ClpB ΔSSD) resulted in a severe defect in oligomerization defect. ClpB ΔSSD stayed predominantly monomeric even in cross-linking experiments. On the other hand we could demonstrate that ATP binding to the first AAA-domain was also necessary for stabilizing the ClpB hexamer. Several observations support this model. First, the Walker A (K212A) and Arg finger (R332A) mutants of the first AAA module did not form stable hexamers in the presence of ATP, whereas corresponding mutations in the second nucleotide binding domain (K611A and R756A) had no influence on ClpB oligomerization, although these mutants exhibited severe structural deficiencies. Second, the introduction of amino acids into the C-domain of the first AAA module (ClpB(Δ410–532) and ClpB ΔLBL) also resulted in destabilization of ClpB oligomers, underlining the importance of this domain in hexamerization. We also could show that high salt conditions (addition of 100 mM (NH₄)₂SO₄) cause dissociation of ClpB oligomers by interfering predominantly with subunit contacts between the first AAA-domains. Interestingly, the recently determined structure of monomeric ClpA suggests that electrostatic interactions between the first AAA-domains of ClpA could play a much more important role than the second AAA modules in protein oligomerization (29). These findings might also explain the observed salt sensitivity of ClpB oligomerization and underline the functional importance of the first AAA-domain in this process. Finally, the observed changes in fluorescence of Trp-543, located in the C-domain of the first AAA module, revealed a conformational rearrangement of this region in response to nucleotides. The observed blue shift (4 nm)
in the wavelength for maximum fluorescence could be attributed to ClpB oligomerization. We suggest that changes in Trp-543 fluorescence reflect interactions of the first C-domain not only with itself but also adjacent ATPase domains, thereby leading to increased shielding of Trp-543 and stabilization of the oligomer. Nucleotide-dependent conformational changes of the C-terminal α-helical domain was also demonstrated by limited proteolysis. Whereas the C-domain was rapidly degraded in the absence of nucleotides, it became largely resistant to proteases upon nucleotide addition (Fig. 5B). Similarly the C-domain of the second AAA module was also protected by addition of nucleotides. This protection is likely to be caused by the interaction of C-domains with their own AAA-domain and that of adjacent subunits, thereby becoming less accessible to proteases. Consistently, ClpB mutants with defects in nucleotide binding (K212A and K611A; 413AAA415) did not exhibit stabilization of the corresponding C-domains.

A functional importance of the first ATPase domain for Hsp100 oligomerization has also been reported for E. coli ClpA (30, 31) and ClpB from Thermus thermophilus (32). It is intriguing that the contributions of the individual AAA-domains differ in the ClpB homologues Hsp104 and Hsp78 from Saccharomyces cerevisiae. Here mutations of the Lys residue in the Walker A motif of the second AAA-domain resulted in a loss of ability to form hexamers (33–35).

Hexamerization of ClpB is a prerequisite for its chaperone activity. Because ATP is bound at the interface of two neighboring subunits in the hexamer, oligomerization additionally influences the ATPase activity of ClpB. Consequently high salt conditions inhibit ATP hydrolysis by ClpB. Furthermore, ClpB variants with defects in oligomerization exhibited little or no ATPase activity. On the other hand, the stimulation of ATP hydrolysis by substrates, such as α-casein, is most likely because of the stabilization of ClpB oligomers.

Mutations of conserved Lys and Glu residues in the Walker A and Walker B motifs of both AAA modules completely abolished ATP hydrolysis by the corresponding AAA-domain. Because single mutants still retained significant ATPase activity, both AAA-domains seem to contribute to the basal rate of ATP hydrolysis by ClpB. Interestingly, ClpB homologues from different organisms differ significantly in their ATPase cycle. Although both AAA-domains of ClpB from T. thermophilus (26, 32) also contribute to the basal ATPase activity, ATP hydrolysis by the yeast homologue Hsp104 is dominated by the first AAA module (25). Such differences may potentially explain the observed species specificity in the cooperation of ClpB/Hsp104 proteins with the corresponding Hsp70 partners (36). Variations in the basal ATPase activity of ClpB mutants indicate communication between both AAA-domains. However, different mutations in the same AAA-domain (E678A and R756A) exhibited different influences on the ATPase activity of the other AAA module. The signaling between both AAA-domains appears to be rather complex, and subtle conformational changes within ClpB mutants can have completely different consequences on the other AAA module.

The function of the N-domain still remains enigmatic. Isolated N-domains from ClpA and ClpB have been shown to form stable, monomeric domains, which are probably separated from the AAA-domains by a flexible linker (7, 37, 38). Consistent with this model, N-domains are not essential for oligomerization of ClpA or ClpB (23, 37, and this work). The connector is apparently sterically inaccessible to proteases because partial proteolysis of ClpB did not release significant amounts of isolated N-domains but rather produced a fragment comprising the N-domain and the core domain of the first AAA module (aa 1–353). Similar findings have been reported for ClpA (37). The reported consequences of N-terminal deletions of ClpB on its chaperone activity are contradictory. Zolkiewski and co-workers (23) showed that a ClpB variant, starting from the internal start site (aa 149–857), was completely inactive in refolding of aggregated luciferase. In contrast, a ClpB ΔN(143–857) variant showed the same chaperone activity as wild type ClpB both in vitro and in vivo (resolubilization of protein aggregates and development of thermotolerance). In agreement with these data, an N-terminal truncated ClpB derivative of Synechococcus sp. PCC7942 conferred the same degree of thermotolerance in vitro as full-length ClpB; likewise, the N-terminal truncated version of T. thermophilus ClpB was also shown to be active in protein disaggregation in vitro (39, 40). The existence of ClpB homologues completely lacking an N-domain in Mycoplasma sp. also argues against an essential function of the N-domains in the disaggregating activities of ClpB (41, 42).

What might be the function of the N-domain with respect to these conflicting results? N-domains have been proposed to mediate substrate binding; however, the reported activities of N-terminally truncated ClpB variants clearly rule out an essential function in this process. Interestingly, defects in substrate binding were also not the basis of the inactivation of ClpB-(149–857), because this deletion variant interacted with casein and unfolded luciferase indistinguishable from ClpB wild type (38). N-domains may therefore be involved in a mechanism for coupling the ATPase cycle of ClpB with its proposed unfolding activity. Because a longer deletion of the N-domain, including parts of the flexible linker to the first AAA-domain, has been reported to be inactive, this linker could potentially contribute to induced structural changes in bound substrates. Alternatively, N-domains may be involved in other ClpB activities, which are so far unknown and are not related to protein disaggregation. Such new activities have been described for a second ClpB homologue in Synechococcus, which is essential for cell viability but is not involved in thermotolerance (43). Interestingly, the N-domain of this ClpB variant seems to be crucial for its unknown activity and may serve as binding sites for special substrates or, alternatively, for specific adaptor proteins. Binding of adaptor proteins to N-domains has been demonstrated for ClpA (44) and the AAA protein p97 (45, 46).

The linker region of ClpB was originally suggested to separate both AAA-domains (1). We propose that the linker instead interrupts the C-domain of the first AAA-domain as was suggested recently (47) for the yeast homologue Hsp104. A comparison of different ClpB fragments (aa 1–409 and 1–567) with respect to their oligomerization and their resistance to proteolysis supports this model. First, cross-linking studies revealed that ClpB-(1–567) can form dimeric species in contrast to the shorter variant (aa 1–409), which remains monomeric under all conditions. Additionally, partial proteolysis revealed a more pronounced protection of the full-length version (aa 1–567), which was not observed for ClpB-(1–409). Formation of dimeric species and the observed partial stabilization is likely to be caused by the interaction of the helical C-domain, which can only be formed in ClpB-(1–567), with its own and an adjacent AAA-domain. Interestingly, a very similar domain organization has been proposed for ClpA (37). In ClpA, which is missing the linker region, a short basic loop (KKRK) is also inserted into the C-domain of the first AAA module.

The linker region of ClpB, in contrast to the N-domain, is essential for chaperone activity. It is proposed to form a 4 times repeated coiled-coil (48), which is very likely to play an important role in ClpB function. Conformational changes in response to nucleotides within the linker region were shown by its increased proteolytic stability in the context of full-length ClpB. We assume that the linker is not an integral part of the ClpB
hexamer, because oligomerization was possible in case of a ClpB variant missing the linker region (ClpB-(Δ410–532)). Similarly, the I-domain of HslU, although inserted into the AAA-domain, forms an independent structural domain and is exposed at the surface of the oligomer (3, 4). The function of the linker region is still unknown. The postulated coiled-coil structure might be involved in protein-protein interaction. Because the ATPase activity of ClpB-(Δ410–532) was still strongly stimulable by casein, the linker region cannot serve as a primary substrate-binding site, at least for this type of substrate.

Alternatively, the linker region is necessary for coupling ATP hydrolysis and substrate unfolding, as proposed recently by Lindquist and co-workers (47).

Acknowledgments—We thank D. Dougan and K. Turgay for discussions and critical reading of the manuscript. We also thank A. Schulze-Specking for technical assistance.

REFERENCES
Roles of Individual Domains and Conserved Motifs of the AAA+ Chaperone ClpB in Oligomerization, ATP Hydrolysis, and Chaperone Activity

Axel Mogk, Christian Schlieker, Christine Strub, Wolfgang Rist, Jimena Weibezahn and Bernd Bukau

doi: 10.1074/jbc.M209686200 originally published online March 6, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M209686200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 26 of which can be accessed free at http://www.jbc.org/content/278/20/17615.full.html#ref-list-1