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The N Terminus of ClpB from *Thermus thermophilus* Is Not Essential for the Chaperone Activity*

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ClpB from *Thermus thermophilus* belongs to the Clp/Hsp100 protein family and reactivates protein aggregates in cooperation with the DnaK chaperone system. The mechanism of protein reactivation and interaction with the DnaK system remains unclear. ClpB possesses two nucleotide binding domains, which are essential for function and show a complex allosteric behavior. The role of the N-terminal domain that precedes the first nucleotide binding domain is largely unknown. We purified and characterized an N-terminal shortened ClpB variant (ClpBΔN; amino acids 140–854), which remained active in refolding assays with three different substrate proteins. In addition the N-terminal truncation did not significantly change the nucleotide binding affinities, the nucleotide-dependent oligomerization, and the allosteric behavior of the protein. In contrast casein binding and stimulation of the ATPase activity by κ-casein were affected. These results suggest that the N-terminal domain is not essential for the chaperone function, does not influence the binding of nucleotides, and is not involved in the formation of intermolecular contacts. It contributes to the casein binding site of ClpB, but other substrate proteins do not necessarily interact with the N terminus. This indicates a substantial difference in the binding mode of κ-casein that is often used as model substrate for ClpB and other possibly more suitable substrate proteins.

ClpB from *Thermus thermophilus* is a member of the AAA protein superfamily that is important for a variety of biological activities (1). Despite their different cellular functions AAA proteins (ATPases associated with a variety of cellular activities) employ a general mechanism. They mediate the assembly and disassembly of large protein complexes that are involved in processes like DNA replication, vesicle transport, or organelle biogenesis. The AAA protein superfamily comprises the Clp/Hsp100 proteins with its members ClpA, ClpX, and ClpY on the one hand and ClpB on the other.

ClpA, ClpX, and ClpY (HslU) interact with cellular peptidases to form ATP-dependent proteases. The role of these Clp proteins is the unfolding of substrates and the delivery of unfolded polypeptides to the protease subunit. In contrast ClpB from *Escherichia coli* and *T. thermophilus* and the *Saccharomyces cerevisiae* homologue Hsp104 do not appear to bind to cellular proteases. Instead they interact with the DnaK/Hsp70 chaperone system to assist the disaggregation of protein aggregates (2–6).

Clp proteins are further classified according to the number of nucleotide binding domains (NBD),[1] which are 220–250 amino acids in length and show a high level of sequence homology. Class two proteins contain only one NBD whereas the class one members ClpA and ClpB contain two NBDs. In ClpB and Hsp104 ATP hydrolysis at both NBDs was shown to be necessary for the chaperone activity of the Clp proteins (7, 8).

Structural information on the class two protein HslU (ClpY) and the hexamerization domain (D2) of *N*-ethylmaleimide-sensitive fusion protein give insight into the domain architecture of AAA proteins (9–11). These crystal structures show ring-shaped oligomers composed of six monomers. The AAA modules of both proteins show a similar overall structure, consisting of the core NBD and a C-terminal, mostly helical, domain. The core NBD contains the Rossman fold including the phosphate binding loop of ATP- and GTP-binding proteins, which consists of five central β-sheets flanked by α-helices. In the case of HslU an additional helical domain is inserted in the NBD that is not present in other Clp proteins.

In $ClpB_{Eco}$ the C-terminal domain of the second AAA cassette was shown to be important for oligomerization, ATPase activity, and chaperone function (12). Isolated C-domains of ClpA, ClpX, and ClpY, which associate with peptidases, were found to interact with different substrate proteins *in vivo*. Therefore the C-terminal domains were proposed to be sensor and substrate discrimination domains although the C-terminal domains show only little sequence homology between different Clp proteins (13).

The class one Clp proteins have, in addition, an N-terminal domain that precedes the first NBD. The ClpA and ClpB mRNAs of *E. coli* contain internal translation initiation sites and are expressed *in vivo* as two gene products: the full-length proteins and shortened versions lacking the N-terminal domain (14, 15). The N-terminal domains are supposed to consist of two ~75-amino acids-long repeats that can fold independently (16, 17). For $ClpA_{Eco}$ it was shown that the N-terminal domain is associated only weakly with the rest of the molecule, and a mutant lacking the N-terminal domain showed a reduced unfolding activity depending on the substrate protein tested (16, 18). Recently an interaction of the N terminus with the proposed ClpA modulator protein ClpS was reported (19).

Similar results were reported for the N-domain of $ClpB_{Eco}$, which did not interact strongly with the shortened ClpB variant (17). The N-terminally shortened ClpB from *E. coli* was

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[1] The abbreviations used are: NBD, nucleotide binding domain; DTE, dithioerythritol; MANT, 2′(3′)-*O*-*N*′-methylanthraniloyl; N-domain, N-terminal domain; C-domain, C-terminal domain; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; LDH, lactate dehydrogenase; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

shown to be inactive as a chaperone but retained its oligomerization capacity and ATPase activity *in vitro* (12). The shortened protein version, however, can restore bacterial thermotolerance *in vivo* (20). In this study we focused on the function of the N-terminal domain of ClpB from *T. thermophilus* and its interactions with substrate proteins. Assigning the individual functions to the different ClpB domains can help to reveal the currently unknown mechanism of protein disaggregation by the ClpB/DnaK system.

EXPERIMENTAL PROCEDURES

Plasmids—The DNA fragment encoding ClpBΔN (amino acids 140–854) was generated by PCR using the pRS-ClpB plasmid as a template (7). The forward primer was GACTGCTAAGCATATGGTGCAGACGGAACACGCGGAAAGC, and the reverse primer was (T7 terminator) CTAGTTATTGCTCAGCGGTGGC. The PCR product was subcloned between the *Nde*I and *Eco*RI restriction sites of *Nde*I/*Eco*RI digested pET27b(+) vector (Novagen). The integrity of the sequence generated by PCR amplification was verified by DNA sequencing.

Proteins—ClpB and ClpBΔN were expressed in *E. coli* BL21(DE3) cells. The purification was performed according to the protocol for the wild type (7). DnaK, DnaJ, and GrpE from *T. thermophilus* were purified as described previously (21). Protein concentration was measured using the method of Ehresmann *et al.* (22). If not noted otherwise all protein concentrations refer to monomers.

Circular Dichroism—CD spectra were recorded 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 millidegrees.

Fluorescence Measurements—Nucleotide affinities were determined using an SLM 8100 photon-counting spectrofluorimeter at 25 °C (7). The fluorescent values were plotted against the ClpB concentration, and the data were analyzed with the quadratic equation using Grafit, Version 3.01 (23). The equilibrium displacements of fluorescent nucleotides by unlabeled nucleotides were fitted to a cubic equation (24).

Binding of ClpB to FITC-casein Type I (Sigma) was monitored using an SLM Aminco Series 2 spectrofluorimeter at 25 °C. The excitation wavelength was 490 nm, and the emission wavelength was 520 nm. Increasing amounts of protein were added to 0.1 mg/ml FITC-casein in 50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM EDTA, 2 mM DTE, 100 mM KCl, 0.1 mg/ml BSA. By varying the excitation wavelength the possibility of light scattering as the reason for the fluorescence increase was excluded.

Chaperone-assisted Reactivation of Substrate Proteins—Recombinant firefly luciferase (Promega) was incubated (10 μM) for 30 min at room temperature in denaturation buffer (25 mM HEPES, pH 7.5, 50 mM KCl, 15 mM $MgCl_2$, 10 mM DTE, 0.05 mg/ml BSA, 1 mM ATP, 7 M urea). For refolding, the denatured luciferase was diluted 125-fold into renaturation buffer (25 mM HEPES, pH 7.5, 50 mM KCl, 15 mM $MgCl_2$, 2 mM DTE, 0.05 mg/ml BSA, 1 mM ATP, 10 mM phosphoenolpyruvate, 240 μM CoA, 0.1 mM luciferin, 50 μg/ml pyruvate kinase) containing 0.5 μM ClpB or ClpBΔN and DnaK (3.2 μM), DnaJ (0.8 μM), GrpE (0.4 μM). The luminescence during refolding was measured continuously at 25 °C over 500 min in microtiter plates with a Fluoroscan *Ascent* FL spectrometer (Labsystems, Helsinki, Finland). Because of product inhibition the luciferase activity declines in the continuous assay after prolonged incubation (25).

α-Glucosidase from *Bacillus stearothermophilus* (Sigma) was used as described previously (4) with the following modifications: α-glucosidase (0.1 μM) was denatured 10 min at 75 °C in the reaction buffer (50 mM MOPS-NaOH, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 1 mM DTE). Chaperones were added (ClpB or ClpBΔN 1.0 μM and DnaK 1.6 μM, DnaJ 0.4 μM, GrpE 0.2 μM) prior to refolding at 55 °C. α-Glucosidase activity was determined at the indicated time points at 40 °C in the assay solution (50 mM sodium phosphate, 2 mM *p*-nitrophenyl-α-D-glucopyranoside) using the iEMS Reader MF microtiter plate reader (Labsystems, Helsinki, Finland).

LDH (0.2 μM) from *B. stearothermophilus* (Sigma) was denatured 30 min at 80 °C in the reaction buffer (50 mM MOPS-NaOH, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 1 mM DTE) (4). Chaperones were added (ClpB or ClpBΔN 1.0 μM and DnaK 1.6 μM, DnaJ 0.4 μM, GrpE 0.2 μM) prior to refolding at 55 °C. LDH activity was determined at the indicated time points at 40 °C in the assay solution (100 mM potassium phosphate, pH 6.0, 0.2 mM NADH, 20 mM sodium pyruvate) using the iEMS Reader MF microtiter plate reader (Labsystems, Helsinki, Finland).

Steady State Kinetics—Steady state ATP hydrolysis was measured with a coupled colorimetric assay (26) using the iEMS Reader MF (Labsystems, Helsinki, Finland) microtiter plate reader (7). ClpB or ClpBΔN (10 μM) was incubated at 25 °C with different concentrations of Mg·ATP in NADH buffer (50 mM Tris/HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 2 mM DTE, 0.4 mM phosphoenolpyruvate, 0.5 mM NADH, 20 μg/ml LDH, 50 μg/ml pyruvate kinase). For casein stimulation κ-casein (Sigma) was added to a final concentration of 0.1 mg/ml. The observed rate constants were determined from the decrease of A_{340} during the first 6 min of incubation. The data were analyzed with Grafit, Version 3.01 using the Hill equation, shown below, describing cooperative binding (7).

$$k = \frac{k_{cat}*[S]^{nH}}{K' + [S]^{nH}} \quad \text{(Eq. 1)}$$

ATPase Stimulation—Steady state ATP hydrolysis was measured in a coupled colorimetric assay (26) using the iEMS Reader MF (Labsystems, Helsinki, Finland) microtiter plate reader (7). ClpB or ClpBΔN (10 μM) was incubated at 25 °C in NADH buffer with 1 mM ATP. Increasing amounts of FITC-casein (Type I; Sigma) or poly-L-lysine (molecular mass 15–30 kDa; Sigma) were added.

Gel Filtration Chromatography—Gel filtration experiments were performed using a Sephadex S200 HR 10/30 column (Amersham Biosciences) with a performance liquid chromatography system (Waters, Milford, MA) (7). ClpB or ClpBΔN (50 μl, 5 mg/ml) was injected with a flow rate of 0.2 ml/min (running buffer: 50 mM Tris/HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM EDTA, 1 mM DTE, 10% glycerol, 1–500 mM KCl). The data from the calibration curve (data not shown) can be fitted to an exponential equation, molecular mass = $Ae^{-k\ R_t}$, where A = 57836.1, and k = 0.10227. The expected retention times (R_t) for the ClpB (molecular mass, 96253 Da) and ClpBΔN (molecular mass, 80904 Da) oligomers were calculated from their molecular mass (kDa) by Equation 2.

$$R_t = \frac{\ln A - \ln \text{molecular mass}}{k} \quad \text{(Eq. 2)}$$

The monomer of ClpB (ClpBΔN) was defined by a molecular mass from 90 to 150 kDa (70–130 kDa), the limits for the dimer were set to 150–240 kDa (130–200 kDa), the limits for the trimer were set to 240–340 kDa (200–290 kDa), and the limits for the higher oligomers were set up to hexamer (*H*) 340–580 kDa (290–530 kDa).

RESULTS

ClpB from *T. thermophilus* shows a high sequence homology to the *E. coli* protein (4). It contains a conserved valine residue (Val-141) encoded by a GUG codon identical to the Val-149 of $ClpB_{Eco}$, where it is used as an internal translation start (14, 27). However, upon expression in *E. coli* cells, no truncated form of the thermophile enzyme was found in significant amounts. To study the properties of the truncated ClpB from *T. thermophilus* we constructed and purified a protein variant lacking the first 139 amino acids, which correspond to the N-terminal domain of $ClpB_{Eco}$ (Fig. 1*A*).

The effect of the N-terminal deletion on the folding of the protein was examined by CD measurements (Fig. 1*B*). The CD spectrum of ClpBΔN is very similar to the spectrum of full-length ClpB. It shows the two negative peaks at 208 and 222 nm, which are characteristic for α-helical proteins. This indicates that the deletion of the N-terminal domain did not influence the folding of the protein significantly and that both proteins are mainly α-helical.

The oligomerization behavior of ClpB and ClpBΔN was determined by gel filtration experiments at 25 °C (Fig. 2). Compared with the full-length protein, ClpBΔN shows a similar oligomerization behavior at the protein concentration used. ATP and low ionic strength induce the formation of higher oligomers up to hexamers whereas the binding of ADP and higher ionic strength favor smaller oligomers. Quantification of the influence of the bound nucleotide and the ionic strength of the buffer allows a direct comparison between the two proteins. The results are summarized in Table I. In the presence of ATP and low ionic strength both proteins form higher oligomers

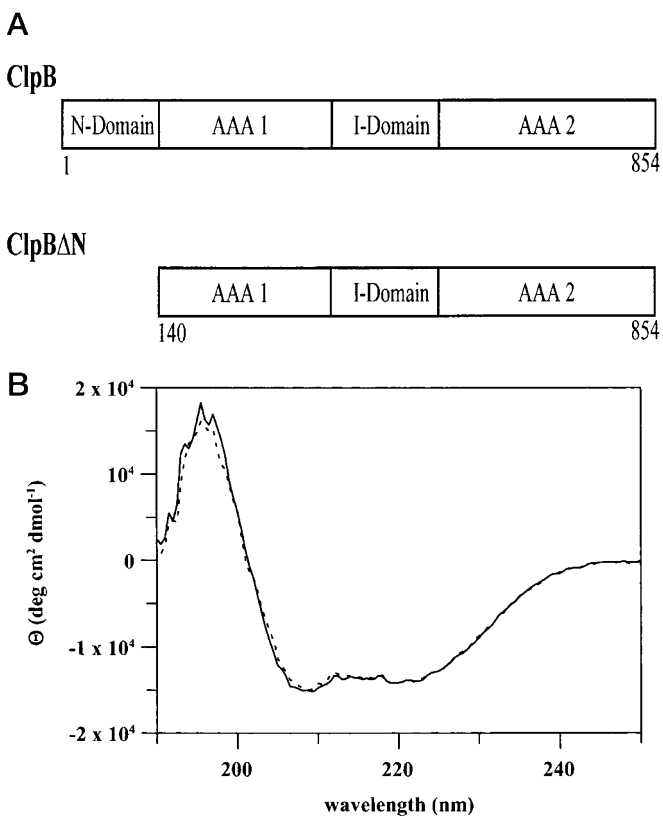

FIG. 1. *A*, structural features of ClpB and ClpBΔN. Shown are the N-terminal domain, the two AAA cassettes, and the intermediate domain between the AAA cassettes. Boundaries were set according to the consensus motifs for AAA+ proteins (1). In ClpBΔN the first 139 amino acids (the N-terminal domain) were removed, and Thr-140 was replaced by Met. *B*, secondary structure of ClpB and ClpBΔN. Far-UV CD spectra of ClpB (*solid line*) and ClpBΔN (*dashed line*) are expressed as mean molar residue elipticity (θ). The spectra were measured at 25 °C in 25 mM potassium phosphate, pH 7.5, 5 mM $MgCl_2$, 50 mM KCl, 5% glycerol.

(1 mM ATP, 1 mM KCl: ClpB, 77.6% hexamer; ClpBΔN, 68.7% hexamer). In the presence of ADP and high salt concentrations the monomer is the preferred oligomeric species (1 mM ADP, 500 mM KCl: ClpB, 88.9% monomer; ClpBΔN, 90.3% monomer). However, ClpBΔN forms more oligomers in the presence of ADP and low to modest ionic strength again compared with ClpB (*e.g.* 1 mM ADP, 200 mM KCl: ClpB, 62.1% monomer; ClpBΔN, 34.7% monomer). These findings demonstrate that the N terminus is not essential for the oligomerization of ClpB in the protein concentration range tested.

To study the influence of the N terminus on nucleotide binding we compared the binding affinities of unmodified nucleotides and the fluorescent nucleotide analogues MANT-ADP and MANT-ATP for ClpB and ClpBΔN at 25 °C (28). The affinities of the MANT nucleotides were determined by equilibrium titration experiments, and the affinities for the unmodified nucleotides were determined by displacement reactions (7). The results are summarized in Table II.

Obviously, the deletion of the N terminus did not influence the nucleotide binding properties significantly. The proteins have almost identical dissociation constants for MANT-ADP (ClpB, 0.2 μM; ClpBΔN, 0.4 μM), and the binding affinity for MANT-ATP is identical (5.4 μM). The unmodified nucleotides ADP and ATP are bound one order of magnitude weaker than the modified nucleotides. The dissociation constants for ADP are again similar (ClpB, 1.5 μM; ClpBΔN, 4.5 μM), and ATP is bound with affinities of 21 μM for the full-length protein and 26 μM for ClpBΔN. As the ATPase activity under the conditions used is very low, the K_d values determined for ATP represent a good approximation and can be compared (7).

To investigate the influence of the N-domain on the ATPase activity, steady state experiments were performed at 25 °C. By plotting the observed rate constants *k versus* the ATP concentration, sigmoidal curves were observed for ClpB and ClpBΔN in the ATP range from 0.1 to 2 mM (Fig. 3). Full-length ClpB shows positive cooperativity of ATP hydrolysis and ATPase stimulation in the presence of κ-casein. ClpBΔN shows a higher basal ATPase activity (k_{cat}, 5.5 min^{-1}), which is stimulated weakly by κ-casein. The data were analyzed with the Hill equation describing cooperative behavior of the proteins (Table III). The k_{cat} of ClpB (3.9 min^{-1}) is increased by 60% when adding κ-casein whereas the k_{cat} of ClpBΔN is only stimulated by ~10%. The k_{cat} in the presence of κ-casein is in a similar range (6.2 min^{-1}) for the two proteins, which might be the maximal ATPase of the hexamer at the given temperature. The allosteric behavior is not changed by the N-terminal deletion, which is reflected by the very similar Hill coefficients (n_H) for the two proteins regarding the unstimulated ATP hydrolysis (ClpB, n_H = 2.4; ClpBΔN, n_H = 2.5) and in the presence of casein (n_H = 3.4 for both proteins). This shows that the N-domain is not involved in forming intermolecular contacts important for the allosteric behavior.

In the next experiments we addressed the question whether the decreased stimulation of ClpBΔN by κ-casein is because of a loss of casein binding or because of reduced coupling of casein binding and ATP hydrolysis. We used fluorescein-labeled casein as an interaction partner of ClpB. First we tested whether this modified casein is able to stimulate the ATPase of ClpB and ClpBΔN similar to κ-casein. ClpB and ClpBΔN were incubated at 25 °C under steady state conditions with increasing concentrations of FITC-casein in the presence of 1 mM ATP (Fig. 4*A*). The ATPase activity of ClpB is stimulated 2.5-fold, and the ATPase of ClpBΔN is only stimulated by 25%. This stimulation is very similar to the stimulation observed by κ-casein (see Fig. 3). Therefore the ATPase activities of ClpB and ClpBΔN respond to FITC-casein as they respond to κ-casein.

The binding of ClpB and ClpBΔN to FITC-casein was monitored by the fluorescence change upon chaperone addition (Fig. 4*B*). Upon addition of ClpB the fluorescence increase (~50%) is more pronounced than after addition of ClpBΔN and reaches a saturated level. The fluorescence increase upon addition of ClpBΔN (~10%) is proportional to the amount of added protein and does not reach saturation. An exact determination of a K_d value was not possible because of the heterogeneous nature of casein and the non-saturated binding of ClpBΔN. It is, however, obvious that ClpBΔN binds FITC-casein with a lower affinity compared with ClpB and that the reduced ATPase stimulation of ClpBΔN by casein is correlating with a reduced binding. To monitor unspecific protein binding, BSA was used as a control.

To examine whether reduced binding affinity and ATPase stimulation of FITC-casein and κ-casein are general features of the truncated ClpB form we used poly-L-lysine, which was shown to stimulate the ATPase of $ClpB_{Eco}$ and Hsp104 (17, 29). ClpB and ClpBΔN were incubated at 25 °C under steady state conditions with increasing concentrations of poly-L-lysine in the presence of 1 mM ATP (Fig. 5). ClpB and ClpBΔN respond to poly-L-lysine very similarly. In both proteins the ATPase activities are stimulated by a factor of 2.5 in the presence of poly-L-lysine. This indicates that the ATPase activity of ClpBΔN can still be stimulated and that both proteins bind poly-L-lysine.

To examine whether the reduced binding affinity for casein is combined with a loss of function we tested the refolding capac-

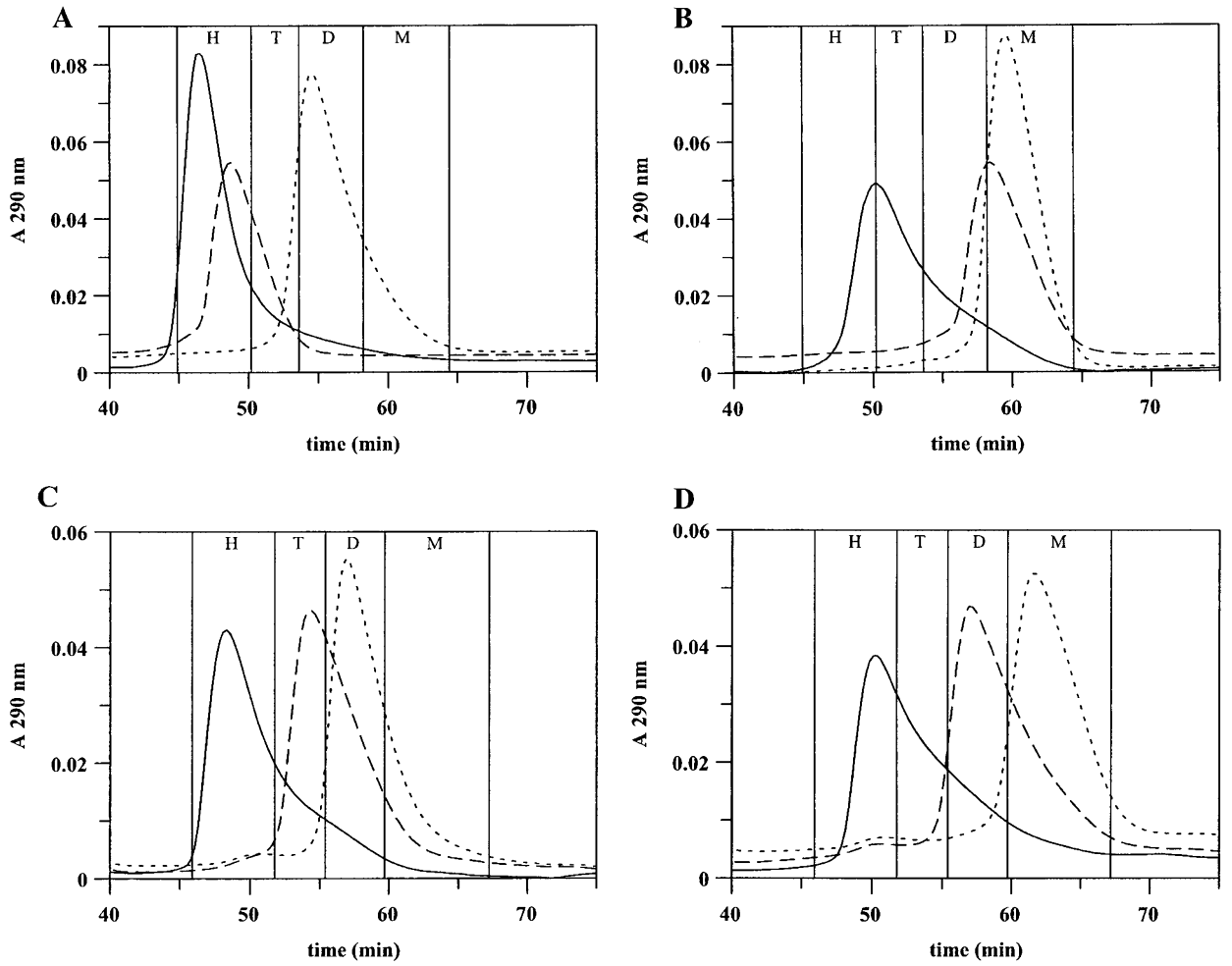

FIG. 2. **Gel filtration analyses.** The oligomerization state of ClpB and ClpBΔN at 25 °C depending on nucleotide and ionic strength was analyzed by gel filtration (see "Experimental Procedures"). ClpB in the presence of ATP (*A*) or in the presence of ADP (*B*) was loaded on a Sephadex S200 HR column in running buffer containing different salt concentrations (1 mM KCl, *solid line*; 200 mM KCl, *dashed line*; 500 mM KCl, *dotted line*). ClpBΔN was analyzed under identical conditions in the presence of ATP (*C*) and ADP (*D*), and different salt concentrations (1 mM KCl, *solid line*; 200 mM KCl, *dashed line*; 500 mM KCl, *dotted line*). *Thin lines* represent boundaries set between monomers (*M*), dimers (*D*), trimers (*T*), and higher oligomers up to hexamers (*H*).

TABLE I
Oligomeric states of ClpB and ClpBΔN

Analysis of the oligomerization state of ClpB and ClpBΔN depending on nucleotide and ionic strength. The values for ClpB are printed bold, and the values for ClpBΔN are given in parentheses. The running buffer contained different concentrations of KCl and 1 mM of the indicated nucleotide. The numbers give the percentage occupancy of a certain molecular weight range for the indicated buffer conditions according to the defined limits (see "Experimental Procedures"). Data for ClpB are taken from Ref. 7.

ATP				
Salt	Monomer	Dimer	Trimer	Tetrahexamer
mM				
1	**3.2** (1.2)	**7.2** (9.9)	**12.0** (20.2)	**77.6** (68.7)
200	**15.3** (10.7)	**39.7** (42.3)	**39.0** (43.9)	**6.0** (3.1)
500	**18.7** (24.4)	**68.9** (68.2)	**12.3** (5.2)	**0.1** (2.2)
ADP				
Salt	Monomer	Dimer	Trimer	Tetrahexamer
mM				
1	**10.4** (7.1)	**24.0** (18.5)	**37.6** (32.4)	**28.0** (42.2)
200	**62.1** (34.7)	**34.2** (57.5)	**2.3** (5.1)	**1.4** (2.7)
500	**88.9** (90.3)	**10.5** (6.6)	**0.5** (1.4)	**0.1** (1.7)

TABLE II
Nucleotide dissociation constants (K_d, μM) for ClpB and ClpBΔN

Binding of unmodified nucleotides and the fluorescent MANT nucleotide analogues to ClpB and ClpBΔN monitored at 25 °C. The affinities of the MANT nucleotides were determined by direct titration experiments, and affinities for the unmodified nucleotides were determined by displacement reactions (see "Experimental Procedures"). Binding of ATP and MANT-ATP was treated as equilibrium binding because of the low intrinsic ATP hydrolysis of the proteins and the relatively short timescale of the experiments. Affinities of ADP and ATP for ClpB are taken from Ref. 7.

	ClpB	ClpBΔN
ADP	1.5	4.5
MANT-ADP	0.2	0.4
ATP	21.0	26.0
MANT-ATP	5.4	5.4

ity of ClpBΔN in cooperation with the DnaK chaperone system. α-Glucosidase, LDH, and luciferase were used as model substrate proteins for the chaperone network. To favor aggregation of the substrates the chaperones were added after the denaturation step. The denatured and aggregated substrate proteins were refolded in the presence of ClpB and the DnaK system, ClpBΔN and the DnaK system, the DnaK system alone, or without chaperones.

α-Glucosidase from *B. stearothermophilus* can be refolded efficiently by the ClpB-DnaK chaperone system at 55 °C after denaturation for 10 min at 75 °C (Fig. 6). The refolding started directly after shifting to 55 °C without a pronounced lag phase. Using full-length ClpB 40% of the original α-glucosidase activ-

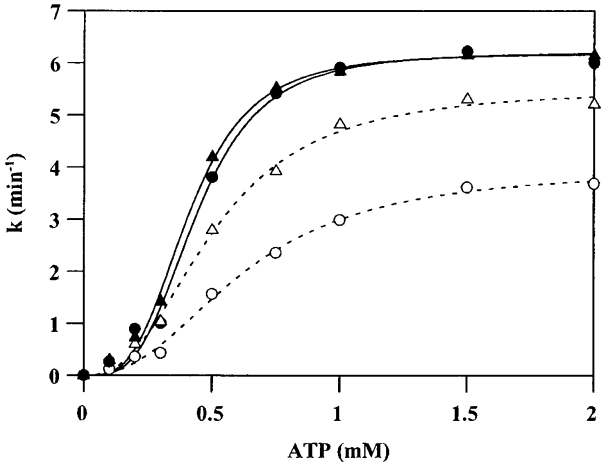

FIG. 3. **Steady state kinetic analysis of ClpB-mediated ATP hydrolysis.** ClpB (○) or ClpBΔN (△) was incubated with different concentrations of Mg·ATP at 25 °C in NADH buffer. For the casein-stimulating effect κ-casein (0.1 mg/ml) was added to the reaction mixture containing ClpB (●) and ClpBΔN (▲). The observed rates (k) for ATP hydrolysis were plotted against the ATP concentration. The data were analyzed using the Hill equation (see "Experimental Procedures").

TABLE III
Kinetic analysis of the steady state ATPase activity using the Hill equation

Steady state experiments with ClpB and ClpBΔN were performed at 25 °C. In the ATP range used (0.1 to 2 mM ATP) a sigmoidal curve is observed for both proteins when plotting the observed rate constant, k versus the ATP concentration. Values obtained without casein are printed bold, and values obtained in the presence of casein are given in parentheses. The data were analyzed using the Hill equation describing cooperative binding (see "Experimental Procedures").

	k_{cat}	K'	Hill coefficient
	min^{-1}	μM	n_H
ClpB	**3.9** (6.2)	**0.32** (0.06)	**2.4** (3.4)
ClpBΔN	**5.5** (6.2)	**0.17** (0.05)	**2.5** (3.4)

ity was restored. ClpBΔN shows ~70% of the wild type activity in this assay. The effect of ClpB is large as shown by the poor refolding efficiency of the DnaK system alone, which is less than 10% of the activity in the presence of ClpB. In the absence of chaperones α-glucosidase did not refold spontaneously to a detectable amount.

LDH from *B. stearothermophilus*, the second substrate protein used, is more stable than α-glucosidase (Fig. 7). After denaturation for 30 min at 80 °C without chaperones less than 5% of the native activity was detected. Both ClpB variants show a similar refolding activity of ~60% of the native LDH after refolding at 55 °C. The effect of ClpB is less pronounced; the DnaK system alone can refold denatured LDH to ~30% of the native activity. After incubation at the refolding temperature without chaperones no increase in LDH activity was detected. Firefly luciferase is a well established substrate of the ClpB-DnaK system (2, 6, 7). We used luciferase as the third substrate protein for the thermophile ClpB-DnaK system (Fig. 8).

Luciferase was denatured for 30 min by urea in the absence of chaperones. For refolding the denatured luciferase was diluted into refolding buffer containing chaperones. In the presence of the DnaK system alone luciferase activity was restored after a lag phase of ~50 min. In the presence of ClpB or ClpBΔN and the DnaK system the renaturation of luciferase started immediately and led to a significantly higher refolding yield. Observation of the luciferase activity showed that no luminescence appeared without chaperones. The activity of

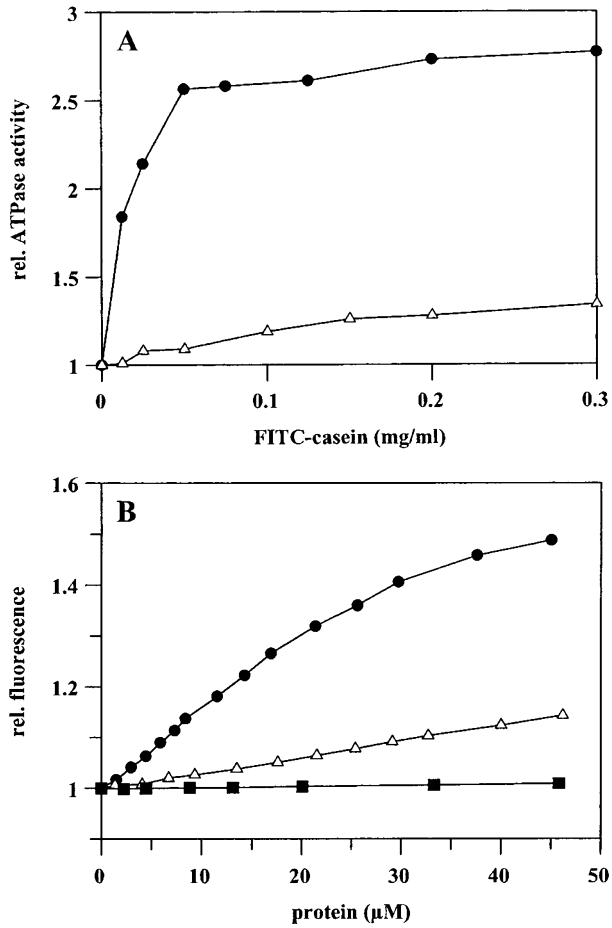

FIG. 4. *A*, ATPase stimulation of ClpB and ClpBΔN by FITC-casein. The observed rates (k) of ClpB (●) and ClpBΔN (△) for ATP hydrolysis at 25 °C were determined as described in the legend for Fig. 3 and set relative to the unstimulated ATPase activity of the proteins. *B*, binding of FITC-casein to ClpB and ClpBΔN. Binding was monitored at 25 °C by the fluorescence intensity of fluorescein-modified casein after addition of ClpB (●), ClpBΔN (△), and BSA (■) (see "Experimental Procedures").

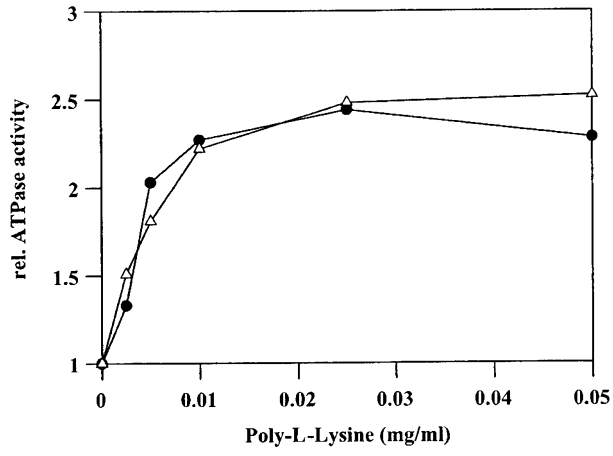

FIG. 5. **ATPase stimulation of ClpB and ClpBΔN by poly-L-lysine.** The observed rates (k) for ATP hydrolysis at 25 °C were determined for ClpB (●) and ClpBΔN (△) as described in the legend for Fig. 3 and set relative to the unstimulated ATPase activity of the proteins.

luciferase declines after prolonged incubation in a continuous assay because of product inhibition (25).

DISCUSSION

The functions of the different domains of Clp proteins have been addressed by a number of studies. Especially the role of

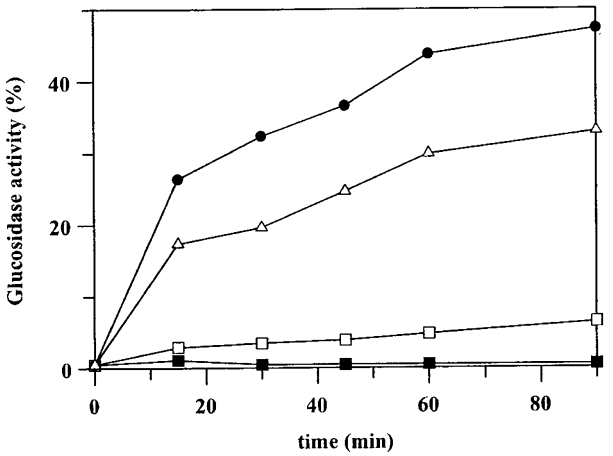

FIG. 6. **Reactivation of α-glucosidase by the ClpB-DnaK system.** Denatured α-glucosidase was reactivated in reaction buffer at 55 °C (see "Experimental Procedures") containing ClpB and the DnaK system ●, ClpBΔN and DnaK system (△), the DnaK system alone (□), or without chaperones (■). α-Glucosidase activity is set relative to the activity before denaturation.

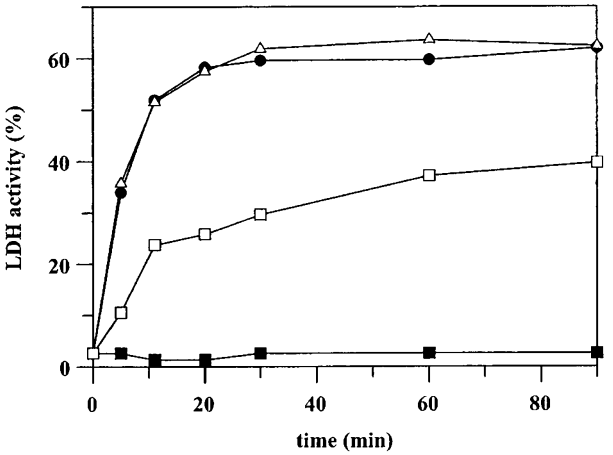

FIG. 7. **Reactivation of LDH by the ClpB-DnaK system.** Denatured LDH was reactivated in reaction buffer at 55 °C (see "Experimental Procedures") containing ClpB and the DnaK system (●), ClpBΔN and the DnaK system (△), the DnaK system alone (□), or without chaperones (■). LDH activity is set relative to the activity before denaturation.

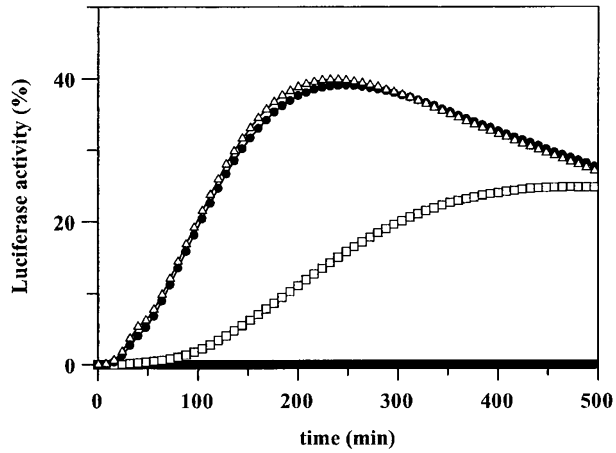

FIG. 8. **Reactivation of luciferase by ClpB DnaK system.** Denatured luciferase was diluted into renaturation buffer (see "Experimental Procedures") containing ClpB and the DnaK system (●), ClpBΔN and the DnaK-system (△), the DnaK system alone (□), or without chaperones (■). Luminescence was detected continuously at 25 °C.

the N terminus was examined because of an internal translation initiation site and the two isoforms of ClpB and ClpA present in *E. coli* (16, 17). Similar to results obtained for $ClpB_{Eco}$ and $ClpA_{Eco}$ the folding of ClpBΔN from *T. thermophilus* is not impaired by the N-terminal deletion. Furthermore we could show that the deletion of the first 140 amino acids did not affect the nucleotide binding properties of the protein, which can be explained by weak interactions of the N-terminal domain with other domains of the molecule, especially with the two NBDs. Weak interactions between the N-domain and the shortened ClpB were also identified in the *E. coli* protein (17).

In addition the N-terminally shortened protein showed a similar oligomerization behavior depending on ion strength and the bound nucleotide. In the presence of ATP and low ionic strength the proteins assemble into higher oligomers, which are stable at 25 °C. An increase of temperature might stabilize these oligomers (30). Therefore the N-domain does not appear to contribute intermolecular contacts necessary for the oligomerization. This implies a position of the N terminus at the outside of the hexameric ring structure. Such a position of the N-domain was also postulated for ClpA from *E. coli* (18). The similar allosteric behavior in steady state ATPase activity of ClpB and ClpBΔN also supports that the N terminus is not involved in oligomerization; both proteins used in this study had very similar Hill coefficients. This confirms that the N-domain is not involved in intermolecular contacts that transmit allosteric signals.

The higher basal ATPase activity of ClpBΔN points at a regulating function of the N-domain. In addition the slightly increased formation of oligomers by ClpBΔN as determined from gel filtration might contribute to the higher ATPase activity.

ClpB shows a stronger stimulation of the ATPase activity by κ-casein compared with ClpBΔN, which is in agreement with results obtained from $ClpB\Delta N_{Eco}$ (12, 31). We were able to demonstrate here that this reduced stimulation of the ATPase activity is because of a reduced binding affinity for casein by ClpBΔN and does not represent a coupling defect of ClpBΔN. The deletion of the N terminus does not generally result in a decreased stimulation of the ATPase activity as poly-L-lysine stimulates ClpB and ClpBΔN equally well. Interestingly, the reduced stimulation of the ATPase activity is restricted to casein, a frequently used model substrate (4, 12, 27).

Considering chaperone activity, the effect of the N-terminal truncation remained obscure. The truncated form was able to restore thermo-tolerance in cyanobacteria *in vivo* (20). In contrast, the N-terminal shortened variant of $ClpB_{Eco}$ was reported to be inactive *in vitro* in a luciferase refolding assay (12). We cannot confirm the *in vitro* result with ClpBΔN from *T. thermophilus*. We observe unaltered refolding capacity for luciferase and LDH and a slightly reduced refolding yield for α-glucosidase. The slightly reduced refolding activity for one substrate protein is in accordance with studies with $ClpA_{Eco}$ (16), which show that the truncated form can still interact with ClpP and degrade substrate proteins albeit at lower rate compared with the full-length ClpA.

Therefore we conclude that the N-terminal domain from ClpB is in general not essential for disaggregation and refolding reactions mediated by the DnaK/ClpB system. It influences the basal ATPase activity and might have an auxiliary function during the interaction with denatured proteins or might only be necessary for a subset of substrate proteins. κ-Casein might belong to this class of substrates that possibly represent a minority. The effects of casein that were used frequently as model substrate for ClpB are therefore not representative for other substrate proteins. This is in agreement with different effects of point mutations in the N-domain of $ClpB_{Eco}$ concern-

ing the ATPase stimulation, which were reported recently (32). Some mutants were no longer stimulated by κ-casein, but all variants tested responded to poly-L-lysine and were still able to bind inactivated luciferase.

Because the N terminus of $ClpA_{Eco}$ was shown recently to interact with the substrate modulator ClpS (19), casein could potentially interact with the binding site of a modulator at the N terminus of ClpB *e.g.* by an unspecific interaction. The effects of the frequently used model substrate casein on ClpB ATPase activity may therefore not be representative for other substrate proteins. In particular as casein is not a well defined single protein species and does not allow for consistent monitoring of refolding. This might represent an increasingly severe problem for the study of ClpB/DnaK disaggregation activity. The substrates (protein aggregates) are in general not well defined, which hampers the direct comparison of mutational effects of ClpB/Hsp104 proteins from different organisms unless sets of substrate proteins are investigated, possibly even including procedures to define the nature of protein aggregates as pioneered recently by Goloubinoff and co-workers (33).

In conclusion, whether the differences between the role of the N terminus from ClpB from *T. thermophilus* and *E. coli* are because of the different experimental procedures or represent true differences of the individual enzymes remains to be shown. The studies presented here with a larger set of substrate proteins including nucleotide binding and hydrolysis studies indicate that the N terminus of ClpB is not essential for *in vitro* chaperone activity.

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