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The N-terminal domain of *Escherichia coli* ClpB enhances chaperone function

I-Ting Chow[a], Micheal E. Barnett[b], Michal Zolkiewski[b], François Baneyx[a,*]

[a] *Department of Chemical Engineering, University of Washington, Box 351750, Seattle, WA 98195, USA*
[b] *Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506, USA*

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Abstract ClpB/Hsp104 collaborates with the Hsp70 system to promote the solubilization and reactivation of proteins that misfold and aggregate following heat shock. In *Escherichia coli* and other eubacteria, two ClpB isoforms (ClpB95 and ClpB80) that differ by the presence or absence of a highly mobile 149-residues long N-terminus domain are synthesized from the same transcript. Whether and how the N-domain contributes to ClpB chaperone activity remains controversial. Here, we show that, whereas fusion of a 20-residues long hexahistidine extension to the N-terminus of ClpB95 interferes with its in vivo and in vitro activity, the same tag has no detectable effect on ClpB80 function. In addition, ClpB95 is more effective than ClpB80 at restoring the folding of the model protein preS2-β-galactosidase as stress severity increases, and is superior to ClpB80 in improving the high temperature growth and low temperature recovery of *dnaK756* Δ*clpB* cells. Our results are consistent with a model in which the N-domain of ClpB95 maximizes substrate processing under conditions where the cellular supply of free DnaK–DnaJ is limiting.

Keywords: Hsp100; Hsp104; ClpB95; ClpB80; Chaperone; Folding; Aggregation

1. Introduction

Escherichia coli ClpA, ClpB, ClpX and ClpY (HslU) are ring-forming members of the Hsp100 family of *h*eat *s*hock *p*roteins (Hsps) which itself is part of the AAA + superfamily (*A*TPases *a*ssociated with various cellular *a*ctivities). AAA + proteins are important for many cellular processes and function by remodeling their substrates in an ATP-dependent manner [1,2]. In *E. coli*, the principal role of ClpA, ClpX and ClpY is to unfold proteins targeted for degradation and to transfer unstructured substrates to an associated proteolytic component [3,4]. By contrast, ClpB collaborates with the DnaK–DnaJ–GrpE (Hsp70) system and the IbpA–IbpB small heat shock proteins to mediate the solubilization and reactivation of aggregated host proteins [5–9].

The crystal structure of *Thermus thermophilus* ClpB [10] has revealed that each ClpB protomer consist of four structural modules: a highly mobile N-terminal domain (N-domain) connected to a first nucleotide binding domain (NBD1) via a flexible linker, a coiled-coil middle (M) domain and a second nucleotide binding domain (NBD2). A model of the ClpB hexamer suggests that the protein is organized in a two-tiered structure with the two NBD domains contributing to oligomerization and located on top of each other in a head to tail arrangement. M domains protrude on the outer surface of the ring and move as rigid bodies. Because disulfide cross-linking of the M domains to NBD1 inactivate ClpB without affecting oligomerization or ATPase activity, it has been proposed that M domains serve as "molecular crowbars" that shear large aggregates into smaller ones [10–12]. However, recent data suggests that like ClpA [13,14] and its *Saccharomyces cerevisiae* homolog Hsp104 [15], *E. coli* ClpB preferentially unfolds protein substrates by threading them through its axial channel [16–18].

In *E. coli*, both the *clpA* and *clpB* transcripts contain conserved internal translation initiation sites that lead to the expression of full-length and N-terminally truncated gene products [19–21]. Although the function of the shortened version of ClpA remains unclear, a small adaptor protein termed ClpS binds to the N-terminal domain of full-length ClpA and redirects ClpAP protease activity from soluble proteins to aggregated species [22–24]. ClpS does not interact with ClpB [24] and no ClpB-specific adaptor protein has been identified to date.

At present, the role of the ClpB N-domain remains controversial. Some studies have found no difference in the ability of the two ClpB isoforms (ClpB95 and ClpB80) to bind model proteins and support the reactivation of aggregated substrates in cooperation with the DnaK–DnaJ–GrpE system [25–27]. Other reports have shown that the N-domain is involved in substrate binding and in coordinating ATP-induced conformational changes in the ClpB core oligomer [19,28–31]. Here, we show that integrity of the N-domain is necessary for optimal ClpB95 function and that ClpB95 is a more effective chaperone than ClpB80 as stress severity increases and availability of the Hsp70 system becomes limiting.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The relevant characteristics of bacterial strains and plasmids used in this study are listed in Table 1. Routine growth was conducted at 30 °C in Luria-Bertani (LB) medium supplemented carbenicillin (100 μg/ml), chloramphenicol (34 μg/ml) and/or spectinomycin (100 μg/ml), as appropriate. Plasmids p95 and p80 were constructed by inserting

*Corresponding author. Fax: +206 685 3451.
E-mail address: baneyx@u.washington.edu (F. Baneyx).

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Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
MC4100	*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	Lab collection
JGT3	MC4100 Δ*clpB::kan*	[39]
JGT20	MC4100 *dnaK756 thr::*Tn*10*	[39]
JGT32	MC4100 *dnaK756 thr::*Tn*10* Δ*clpB*::*kan*	[39]
JGT6	MC4100 *zjd::*Tn*10 groES30*	[39]
JGT38	MC4100 *zjd::*Tn*10 groES30* Δ*clpB::kan*	[39]
Plasmids		
pMM103	pACYC184 derivative encoding the *trc* promoter, a multiple cloning site and the *rrnB* terminator (Chl^r)	[32]
pClpB95/80	pMM103 derivative encoding *clpB95*/*clpB80* under *trc* transcriptional control (Chl^r)	[32]
pClpB95	pMM103 derivative encoding *clpB95* under *trc* transcriptional control (Chl^r)	[32]
pClpB80	pMM103 derivative encoding *clpB80* under *trc* transcriptional control (Chl^r)	[32]
pSR22	pBR322 derivative in which the *Nco*I site was exchanged to *Nde*I by site-directed mutagenesis (Amp^r)	[32]
pGC1	pSR22 derivative encoding the *trc* promoter, a polyhistidine tag and a multiple cloning site (Amp^r)	[32]
pH95/80	pGC1 derivative encoding a hexahistidine-tagged version of *clpB95*/*clpB80* under *trc* transcriptional control (Amp^r)	[32]
pH95	pGC1 derivative encoding a hexahistidine-tagged version of *clpB95* under *trc* transcriptional control (Amp^r)	[32]
pH80	pGC1 derivative encoding encoding a hexahistidine-tagged version of *clpB80* under *trc* transcriptional control (Amp^r)	[32]
p95	pSR22 derivative encoding *clpB95* under *trc* transcriptional control (Amp^r)	This work
p80	pSR22 derivative encoding *clpB80* under *trc* transcriptional control (Amp^r)	This work
pMS421	pSC101 derivative encoding *lacI*^{q} (Spc^r)	Kelly Hughes (UW)
pTBG(H^-)	pBR322 derivative encoding PreS2-β-galactosidase under *tac* transcriptional control (Amp^r)	[47]

Abbreviations are: Amp, ampicillin; Chl, chloramphenicol; Spc, spectinomycin; r superscript, resistant.

*Nde*I-*Xho*I fragments isolated from pClpB95 and pClpB80 and encoding *clpB95* and *clpB80*, respectively, into pSR22 digested with the same enzymes.

2.2. Cell viability and growth studies

The cell viability experiments of Fig. 1A were conducted as described in the accompanying paper [32]. For the experiments of Fig. 3A, aliquots from overnight cultures were used to inoculate 25 mL of LB medium supplemented with spectinomycin and carbenicillin and prewarmed to 42.5 °C to an initial OD_{600} reading of 0.05. Flasks (125 mL) were incubated at 42.5 ± 0.2 °C in a New Brunswick G76 water bath and OD_{600} readings were taken at 30 min intervals. For the experiment of Fig. 3B, flasks were transferred to 30 °C after 90 min incubation at 42.5 °C and the OD_{600} of the cultures was recorded at 30 min intervals for an additional 4.5 h. For the spot test experiments of Fig. 3C, JGT32 harboring pMS421 and either pClpB95/80, pClpB95, pClpB80 or pMM103 were grown overnight at 30 °C in LB supplemented with spectinomycin and chloramphenicol. JGT20 (pMS421 + pMM103) cultures were used as a control. The OD_{600} of the samples was adjusted to 1.0 and dilutions (10^{-1}–10^{-5}) were made with LB. Aliquots (10 μl) of the dilutions were spotted on LB plates supplemented with spectinomycin and chloramphenicol. Plates were incubated overnight at 30 °C or first held at 42.5 °C for 2 or 4 h before transfer to 30 °C.

2.3. PreS2-β-galactosidase folding

JGT3 cells harboring pMS421, pTBG(H^-) and either pMM103, pClpB95/80, pClpB95 or pClpB80 were grown at 30 °C in LB medium supplemented with chloramphenicol, spectinomycin and carbenicillin to $OD_{600} \sim 0.2$. Cultures were treated with 1 mM IPTG to induce recombinant protein production, grown to $OD_{600} \sim 0.4$ and transferred to baths held at 42 or 45 °C. Samples collected after 1 h were fractionated into soluble and insoluble fractions and assayed for β-galactosidase activity [33]. All experiments were performed in triplicate. Activities are reported in Miller units ($1000 \times \Delta\ OD_{420}/OD_{600}$ of culture per ml of culture per min of reaction) and error bars correspond to independent triplicate experiments. Differences in final OD_{600} between pClpB transformants were less than 5%.

2.4. ATPase activity assays

Native ClpB95 or its N-terminally His-tagged variant (His-ClpB95) were incubated for 15 min at 37 °C in 100 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM ATP, 1 mM EDTA, 1 mM DTT, in the absence or in the presence of 0.1 mg/ml κ-casein (Sigma). Inorganic phosphate concentration was measured as previously described [9] in 50-μl samples containing 2.5 μg ClpB95 without casein, 0.5 μg ClpB95 with casein, 5 μg HisClpB95 without casein, or 3 μg His-ClpB95 with casein.

3. Results

3.1. His-tagging of the N-domain interferes with ClpB95 activity

When overproduced at comparable concentrations, ClpB95 and ClpB80 are almost equally efficient at protecting Δ*clpB* cells from thermal killing at 50 °C [32]. To determine if ClpB95 requires an intact N-domain to exhibit its protective function, we constructed a series of ColE1 derivatives in which genes encoding native *clpB*, *clpB95* and *clpB80* under *trc* transcriptional control were fused or not to a 20-codon 5′ extension specifying a hexahistidine sequence and a thrombin cleavage