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Cooperation between two ClpB isoforms enhances the recovery of the recombinant β-galactosidase from inclusion bodies

Izabela Guenther [a], Michal Zolkiewski [b], Sabina Kędzierska-Mieszkowska [a,*]

[a] Department of Biochemistry, University of Gdansk, Wita Stwosza 59, 80-308 Gdansk, Poland
[b] Department of Biochemistry, Kansas State University, Manhattan, KS 66506, USA

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ABSTRACT

Bacterial ClpB is a molecular chaperone that solubilizes and reactivates aggregated proteins in cooperation with the DnaK chaperone system. The mechanism of protein disaggregation mediated by ClpB is linked to translocation of substrates through the central channel within the ring-hexameric structure of ClpB. Two isoforms of ClpB are produced *in vivo*: the full-length ClpB95 and the truncated ClpB80 (ClpBΔN), which does not contain the N-terminal domain. The functional specificity of the two ClpB isoforms and the biological role of the N-terminal domain are still not fully understood. Recently, it has been demonstrated that ClpB may achieve its full potential as an aggregate-reactivating chaperone through the functional interaction and synergistic cooperation of its two isoforms. It has been found that the most efficient resolubilization and reactivation of stress-aggregated proteins occurred in the presence of both ClpB95 and ClpB80. In this work, we asked if the two ClpB isoforms functionally cooperate in the solubilization and reactivation of proteins from insoluble inclusion bodies (IBs) in *Escherichia coli* cells. Using the model β-galactosidase fusion protein (VP1LAC), we found that solubilization and reactivation of enzymes entrapped in IBs occurred more efficiently in the presence of ClpB95 with ClpB80 than with either ClpB95 or ClpB80 alone. The two isoforms of ClpB chaperone acting together enhanced the solubility and enzymatic activity of β-galactosidase sequestered into IBs. Both ClpB isoforms were associated with IBs of β-galactosidase, what demonstrates their affinity to this type of aggregates. These results demonstrate a synergistic cooperation between the two isoforms of ClpB chaperone. In addition, no significant recovery of the β-galactosidase from IBs in *ΔclpB* mutant cells suggests that ClpB is a key chaperone in IB protein release.

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1. Introduction

Many kinds of environmental stress, such as a sudden increase of temperature, the presence of ethanol or heavy metals cause intracellular denaturation and aggregation of proteins both in prokaryotic and eukaryotic cells [1–3]. A common feature of these stresses is their ability to perturb protein structure ultimately leading to protein aggregation. The presence of protein aggregates in living cells induces the heat-shock response and the rapid synthesis of a specific group of proteins, the called heat shock proteins (*Hsps*) or stress proteins [4–6]. Among Hsps, there are molecular chaperones including the two major chaperone systems: DnaK/DnaJ/GrpE, GroES/GroEL and proteases such as ClpAP, HtrA or Lon. Molecular chaperones facilitate the proper folding of polypeptides, protect other proteins from thermal inactivation, and reactivate aggregated proteins. Heat shock proteases eliminate proteins irreversibly damaged by stress.

Protein aggregation is also frequently observed upon biosynthesis of heterologous proteins in *Escherichia coli*, which is the most commonly used organism for production of recombinant proteins. Overproduction of these proteins is often accompanied by their deposition into insoluble inclusion bodies (IBs).

During the past decade, many studies have been focused on the disaggregation and reactivation of proteins by a bi-chaperone system consisting of DnaK/DnaJ/GrpE and ClpB, a member the Hsp100/Clp subfamily of AAA+ ATPases (ATPases Associated with a variety of cellular Activities) [7–12]. Like other Hsp100 proteins, ClpB forms barrel-shaped hexamers in the presence of ATP [13]. Each ClpB protomer is composed of an N-terminal domain, two AAA+ ATP-binding modules (NBD-1, NBD-2), and an inserted coiled-coil middle domain (Fig. 1). Yeast proteins Hsp104 (located in the cytosol) and Hsp78 (found in the mitochondrial matrix), and plant Hsp101 are among the eukaryotic homologues of the bacterial ClpB.

The *E. coli clpB* gene contains an internal translation–initiation site and is expressed *in vivo* as two proteins: a full-length 95-kDa ClpB (ClpB95) and a N-terminal-truncated 80-kDa isoform (ClpB80,

* Corresponding author. Fax: +48 58 5236309.
E-mail address: kedzie@biotech.ug.gda.pl (S. Kędzierska-Mieszkowska).

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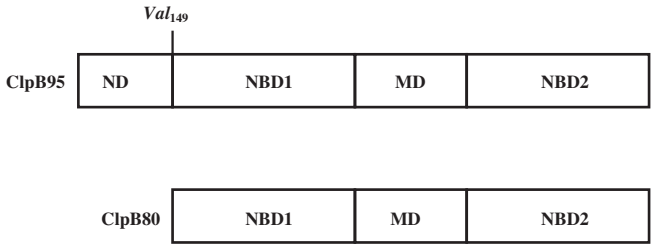

Fig. 1. Domain organization of one monomer of ClpB95 and ClpB80. The diagram shows the structural domains of the two ClpB isoforms: N-terminal domain (ND), nucleotide binding domains 1 (NBD1), middle coiled-coil domain (MD) and nucleotide binding domain 2 (NBD2). Only ClpB95 contains a 149-residue-long N-terminal domain.

ClpBΔN) [14] (Fig. 1). The internal initiation site is located near the N-terminus of the first nucleotide-binding domain in ClpB. Thus, the N-terminal domain of ClpB95 is not present in ClpB80. The biological role of the truncated isoform in reactivation of stress-aggregated proteins has been discovered only recently [15]. It was found that ClpB95 and ClpB80 cooperate in reactivation of proteins aggregated upon stress conditions to produce a highly efficient chaperone system. It is assumed that the functional cooperation between ClpB isoforms arises from interactions between them, because ClpB95 and ClpB80 associate into hetero-oligomers, which form with a higher efficiency than the homo-oligomers of ClpB95. Thus, it is very likely that the ClpB95/80 hetero-association boosts the aggregate-reactivation potential of ClpB and *E. coli* produces two isoforms of ClpB to optimize its activity. The purpose of this work was to investigate futher the role of ClpB95 and ClpB80 in the mechanism of protein disaggregation and reactivation. We examined an effect of ClpB95/ClpB80 on the solubility and activity of the VP1-β-galactosidase (VP1LAC) accumulating as IBs in the cytoplasm of *E. coli* cells. The obtained results support a recent finding that disaggregation and reactivation of aggregated proteins require synergistic cooperation of the two isoforms of ClpB and provide supplementary data about the general role of this chaperone in protein disaggregation.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth condition

E. coli MC4100 (SG20250) (*ara*D139, Δ(*argF-lac*)U169, *rpsL*150, *rel*A1, *deo*C1, *pts*F25, *rps*R, *flb*B53010), used as wild-type (wt), was obtained from S. Gottesman (National Cancer Institute, Bethesda, MD), and its derivative MC4100*ΔclpB*::kan was supplied by A. Toussaint (Université Libre de Bruxelles, Brussels, Belgium). Plasmid pJCO46 and pJVP1LAC were kindly provided by A. Villaverde (Institut de Biologia Fonamental and Departament de Genetica and Microbiologia, Universitat Autonoma de Barcelona, Spain) [16]. Plasmids pClpB95/80, pClpB95, pClpB80 (pGB2-derivatives) carrying an appropriate *clpB* variant together with the σ^{32}-dependent promoter were constructed earlier [15,17]. Plasmid pGB2 (spc^R, str^R) was used as a control [18]. DNA plasmid preparation and transformation of *E. coli* strains were done according to [19].

Bacterial cultures were grown in Luria–Bertani (LB) medium supplemented with 100 μg/ml ampicillin, 30 μg/ml kanamycin, and 50 μg/ml spectinomycin, as appropriate, at 28 °C to OD_{600} = 0.3. Then, bacteria were transferred to 42 °C to induce both the recombinant protein and ClpB chaperone production. After 2 h of thermal induction, protein synthesis was arrested by chloramphenicol addition at 200 μg/ml and the cultures were further incubated at 28 °C for 3 h to examine the ability of ClpB isoforms to resolubilization and reactivation of β-galactosidase sequestered into IBs. Culture samples (containing equal amounts of bacteria) collected at different time points after temperature up-shift and during the period of growth at 28 °C were assayed for the β-galacosidase activity and separated into the soluble and insoluble protein fractions.

2.2. Separation of soluble and insoluble protein fractions and IB purification

The bacterial cells were disrupted by a non-mechanical lysis method that utilized the B-PER bacterial protein extraction reagent (Pierce). The preparation of the soluble and insoluble protein fractions was performed according to the manufacturer's instruction. Briefly, each cell pellet from 1 ml of bacterial culture was mixed with 300 μl of B-PER solution, and the mixture was vortexed for 1 min. Next, the soluble fraction was obtained from the supernatant by microcentrifugation at 12,000*g* for 10 min, and the insoluble fraction was prepared by resuspension of the disrupted pellet in 300 μl of B-PER solution. Six microliters of lysozyme solution (10 mg/ml) was subsequently added to that suspension, which was again mixed for 1 min. Then 1 ml of B-PER reagent diluted 20-fold was added and the suspension was further mixed for 1 min. The purified IBs were collected by microcentrifuge at 12,000*g* for 15 min. Finally, the purified IBs were prepared by three more washes with B-PER reagent diluted 20-fold and then resuspended in 300 μl of B-PER solution B-PER reagent. VP1LAC fusion protein in the obtained fractions was analyzed by Western Blotting followed by a densytometry.

2.3. Analytical methods

β-Galactosidase activity was determined according to Miller's method [20] and its activity is reported in Miller units ($1000 \times OD_{420}/OD_{600}$ of culture per ml of culture per min of reaction). Proteins from the soluble and insoluble IB fractions were resolved by SDS–PAGE [21], then Western Blotting was performed as described [22]. Polyclonal rabbit antisera against β-galactosidase or ClpB were used and the reactions were developed using the goat anti-rabbit horseradish peroxidase conjugate (Sigma), and 3,3′-diaminobenzidine (Sigma), and H_2O_2 as substrates. Membranes were scanned and analyzed with 1Dscan EX, Scananalytics Inc. Sigma program.

3. Results and discussion

To explore the participation of ClpB80 and ClpB95 in the resolubilization and reactivation of the VP1-β-galactosidase aggregating as IBs, we used the *E. coli* strain MC4100*clpB*+ (wt) and its derivative MC4100*ΔclpB*. The *ΔclpB* mutant strain was transformed with a plasmid encoding both ClpB95 and ClpB80, ClpB95 or ClpB80 (ClpBΔN) alone, and also plasmid pJVP1LAC. The wild type strain was transformed with pJCO46 or pJVP1LAC and used as controls. pJCO46 encodes a soluble, pseudo-wild type *E. coli* β-galactosidase, and its derivative pJVP1LAC, a hybrid protein in which the aggregation-prone VP1 capsid protein of the foot-and-mouth disease virus is fused to the N-terminus of β-galactosidase [16]. The expression of both *lacZ* and *VP1LAC* genes is under the control of tandem λ *pLp*R lytic promoters and is repressed by a temperature-sensitive CI857 repressor. The total protein levels of ClpB95/80, ClpB95, and ClpB80 were comparable in these experiments (see Fig. 4, below).

When monitoring the β-galactosidase activity inside the bacterial cells, we noticed that the enzymatic activity of the non-aggregating β-galactosidase (produced from the plasmid pJCO46), was nearly fivefold higher after 2 h incubation at 42 °C than the activity of the VP1LAC fusion protein (Fig. 2). For the β-galactosidase aggre-

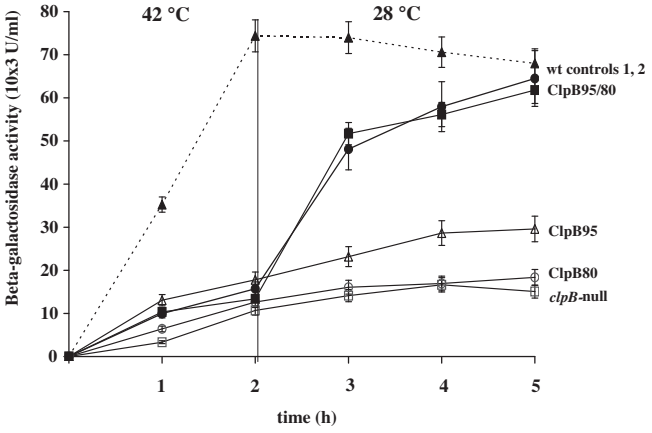

Fig. 2. Activity of β-galactosidase in *E. coli* cells. *E. coli* cultures producing either the wild type β-galactosidase or VP1LAC fusion protein were grown at 42 °C for 2 h. Then chloramphenicol was added and the cultures were incubated further at 28 °C for 3 h (recovery period). At the indicated time culture aliquotes were taken and assayed for β-galactosidase activity. The data are shown for the *ΔclpB* mutant carrying the control plasmid pGB2, the strains producing simultaneously both ClpB95 and ClpB80, ClpB95 or ClpB80, and wt strain transformed with pJCO46 (control 1, triangles) or pJVP1LAC (control 2, circles). Error bars represent the standard deviation of three experimental values. The vertical line indicates the addition of chloramphenicol and temperature down-shift.

gated in IBs the loss of enzymatic activity ranged from 70% to 80% as compared to the activity of wild type enzyme. Moreover, the activity of wild type β-galactosidase slowly decreased after the arrest of protein synthesis by chloramphenicol addition (Fig. 2, wt control 1). This decrease in the enzymatic activity of the soluble β-galactosidase may arise from its degradation by cellular proteases what was earlier suggested [23]. In the case of the insoluble VP1LAC protein, in all tested strains, the enzymatic activity increased proportionally with the incubation time at 28 °C (Fig. 2). After 3 h incubation, the highest activity of the IB-sequestered β-galactosidase (~62,000 U/ml) was detected in bacterial cells producing simultaneously both ClpB95 and ClpB80. It is worthy to note, that there is no significant difference between the β-galactosidase activity regain in the control strain, MC4100*clpB*+, and MC4100*ΔclpB* carrying pClpB80/95, therefore the wild type strain was omitted in further experiments. In the presence of ClpB95 alone, the enzyme activity was only 50% of that found with ClpB95/80 (~30,000 U/ml). The ClpB80 isoform alone did not significantly influence the reactivation of insoluble β-galactosidase. The enzymatic activity, in this case, was similar to that obtained with MC4100*ΔclpB*[pGB2]. These results may suggest that the efficient reactivation of enzymes trapped into IBs requires the simultaneous presence of both ClpB isoforms. The reactivation yield in the presence of ClpB95 with ClpB80 was higher than the sum of the yields of the two ClpB isoforms working independently. These observations agree with the previous studies [15] and again point to the importance of cooperation between the two ClpB isoforms in aggregate reactivation. In conclusion, ClpB95 and ClpB80 work together to produce a highly efficient aggregate-reactivating chaperone system.

Next, we estimated the percentage content of VP1LAC in the soluble protein fraction and insoluble IBs in relation to its total amount in both fractions (set as 100%). For this purpose, cultures of *E. coli* MC4100*ΔclpB* producing IBs of β-galactosidase and carrying either pGB2 (control), pClpB95/80, pClpB95 or pClpB80 were used for protein fractionation. The soluble protein fraction and insoluble IBs were analyzed by 0.1%SDS–7.5% PAGE followed by Western Blotting using anti-β-galactosidase antibodies (data not shown). Immunoreactive band corresponding to the VP1LAC protein was analyzed with Sigma Gel software. Based on the densyto-

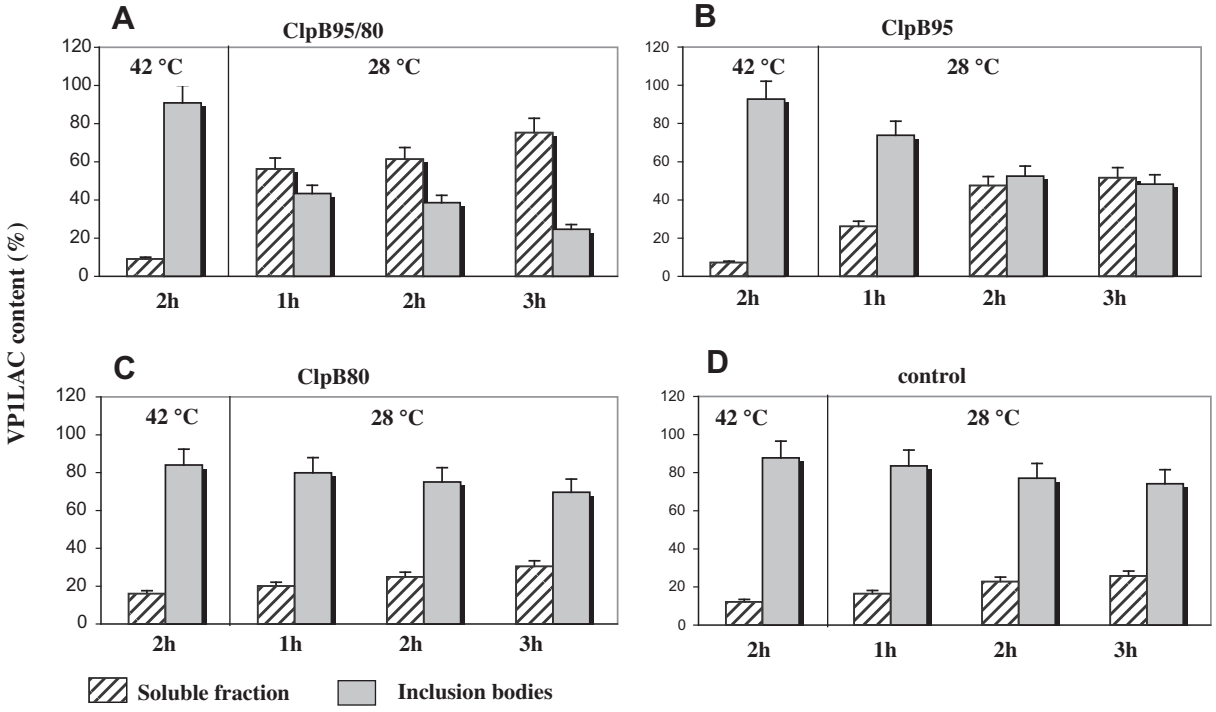

Fig. 3. Distribution of VP1LAC in the soluble and insoluble IB fractions from *E. coli* cells producing ClpB95 and/or ClpB80. The baterial cultures were grown at 42 °C for 2 h, then chloramphenicol was added to block the *de novo* protein synthesis. For subsequent recovery, bacteria were incubated further at 28 °C for 3 h. At the indicated time culture samples were collected, lysed and fractionated. Proteins from the soluble and insoluble IB fractions were analyzed by SDS–PAGE. β-Galactosidase antiserum was used for VP1LAC immunodetection and estimation by densitometry. *ΔclpB* mutant cells carrying pGB2 was shown as a control panel. Results are presented as the average of three independent experiments with standard deviations indicated. The amount of sample loaded onto the gel was estimated by measurements of the optical density at 600 nm of bacterial cultures.

metric measurements, we defined a participation of ClpB isoforms in the resolubilization of IBs (i.e. in enzyme release from IBs). Results presented in Fig. 3A showed that the *E. coli* cells producing both ClpB isoforms achived the fastest rate of resolubilization of β-galactosidase from aggregates and after 3 h incubation at 28 °C ~90% of the enzyme appeared in the soluble protein fraction. In the cells transformed with pClpB95, at the same time, ~50% of VP1LAC fusion protein still remained in aggregates (Fig. 3B). In the presence of ClpB80 only ~30% of the β-galactosidase was found in the soluble fraction, the rest of enzyme was aggregated in the form of IBs (Fig. 3C). A similar result was obtained for the control strain, MC4100*ΔclpB*[pGB2], in this case 25% of VP1LAC protein was in the soluble form after 3 h of recovery (Fig. 3D). These results suggest that the lack of ClpB function causes a significant decrease in the amount of soluble VP1LAC as compared to that produced by the strain carrying pClpB95/80. In the mutant *ΔclpB*, VP1LAC protein was mainly in insoluble IBs. This observation indicates that ClpB chaperone plays a key role in IB protein release. Its action in the disintergation of IBs would be assisted by co-chaperone DnaK and small heat-shock proteins, IbpA/B. The presence of ClpB80 alone did not result in the resolubilization of VP1LAC aggregates and the fusion protein was mostly found in IBs. This result shows that ClpB80 alone is not able to solubilize IBs of β-galactosidase. ClpB95 was more effective in this process than its truncated isoform ClpB80. However, when both ClpB95 and ClpB80 were present simultaneously in the cells, the most efficient resolubilization of β-galactosidase from IBs was observed. The results of densytometric analyses correspond to measurements of the β-galactosidase activity in *E. coli* cells (Fig. 2). As the β-galactosidase activity increased, we observed, in all tested strains, a reduction in the insoluble VP1LAC fraction and a gradual increase in its content in the soluble protein fraction (Fig. 3), but only ClpB95 and ClpB80 acting together were able to significantly increase the amount of soluble enzyme and enhance its activity.

A recent study has shown that both ClpB95 and ClpB80 were components of the fraction containing endogenous proteins of *E. coli* aggregated upon heat-shock [15]. It was also demonstrated that DnaK and GroEL chaperones or sHsps are associated with IBs [24–27]. We therefore tested whether ClpB isoforms may be also associated with exogeneously formed IBs. To examine this possibility, we performed immunodetection of ClpB95 and ClpB80 in the soluble protein fraction and insoluble IBs using anti-ClpB serum (Fig. 4). For immunoblotting analysis, the culture samples collected after 2 h incubation at 42 °C were analyzed. As shown in Fig. 4, we found both ClpB95 and ClpB80 in the purified IBs. However, the majority of these proteins were detected in the soluble fractions. Localization of both ClpB isoforms in VP1LAC aggregates could suggest that ClpB95 as well as ClpB80 has an affinity to such type of aggregates as IBs. Obviously, the association of ClpB with VP1LAC aggregates could result in unspecific interactions with IBs during purification process. However, taking into account the function of ClpB chaperone, its dominant role in solubilization of insoluble sHsps/substrate complexes [28], a specific co-aggregation of ClpB with a recombinant protein that forms IBs is very likely. The data in Fig. 4 also show that whereas ClpB80 bound to the insoluble fraction as efficiently as ClpB95, it showed lower rate of resolubilization and reactivation of VP1LAC. Thus, binding of the chaperone lacking the N-terminal domain to the aggregates is not sufficient for their reactivation. Moreover, our results show that a relatively small amount of ClpB80 bound to the aggregates together with ClpB95 (see Fig. 4) synergizes with the full-length isoform and supports an efficient IB resolubilization.

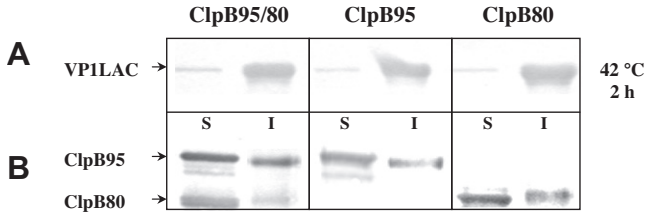

Fig. 4. Identification of ClpB isoforms in the insoluble IBs of β-galactosidase. Immunodetection of VP1LAC protein (A) and ClpB95/80 (B) in the soluble (S) and insoluble IB (I) fractions obtained from *E. coli ΔclpB* cells producing ClpB95 and/or ClpB80, and also VP1LAC fusion protein collected after 2 h incubation at 42 °C. The amount of sample loaded onto the gel was estimated by measurements of the optical density at 600 nm of bacterial cultures.

In summary, our data indicate that the reactivation yield and solubility of recombinant proteins aggregated in form of IBs in *E. coli* cells are significantly increased by the simultaneous presence of both ClpB95 and ClpB80. This result supports an important role of synergistic cooperation between the two ClpB isoforms in aggregate reactivation [15] extends previous observations into the reactivation of IBs. Protein aggregation and formation of IBs is a major impediment in biochemical research and biotechnology. Understanding how nature uses a molecular machine, like ClpB, to reverse protein aggregation, may help in the future to design of strategies to employ chaperones as aggregation-controlling factors and to obtain reliable methodologies of recombinant protein production and purification.

Acknowledgments

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