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Role of the disaggregase ClpB in processing of proteins aggregated as inclusion bodies

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ABSTRACT

Overproduction of heterologous proteins in bacterial systems often results in the formation of insoluble inclusion bodies (IBs), which is a major impediment in biochemical research and biotechnology. In principle, the activity of molecular chaperones could be employed to gain control over the IB formation and to improve the recombinant protein yields, but the potential of each of the major bacterial chaperones (DnaK/J, GroEL/ES, and ClpB) to process IBs has not been fully established yet. We investigated the formation of inclusion bodies (IBs) of two aggregation-prone proteins, VP1LAC and VP1GFP, overproduced in *Escherichia coli* in the presence and absence of the chaperone ClpB. We found that both ClpB isoforms, ClpB95 and ClpB80 accumulated in *E. coli* cells during the production of IBs. The amount of IB proteins increased in the absence of ClpB. ClpB supported the resolubilization and reactivation of the aggregated VP1LAC and VP1GFP in *E. coli* cells. The IB disaggregation was optimal in the presence of both ClpB95 and ClpB80. Our results indicate an essential role of ClpB in controlling protein aggregation and inclusion body formation in bacteria.

Introduction

Protein aggregation is caused by a number of factors, which lead to the accumulation of unfolded or partially folded polypeptides. A common type of protein aggregates are inclusion bodies (IBs)[1], which are often formed in *Escherichia coli* cells as a result of high-level expression of genes encoding heterologous proteins [1]. Aggregation of recombinant proteins during their overproduction results from an abrupt increase in concentration of newly synthesized polypeptides. It has been postulated that an excessive rate of biosynthesis of heterologous proteins results in a rapid depletion of the available pool of molecular chaperones that support *de novo* folding of polypeptides. Thus, overproduction of recombinant proteins is likely to overload the cellular quality-control machinery. The chaperone overload results in a local accumulation of partially folded or misfolded protein conformations, which favors the formation of protein aggregates, mostly stabilized by intermolecular hydrophobic interactions. The uncontrolled aggregation of recombinant proteins in *E. coli* cells and formation of IBs is a major impediment in biochemical research, biotechnology and biomedicine. In principle, the activity of molecular chaperones could be employed to gain control over the IB formation and to improve the recombinant protein yields. The potential of each of the major bacterial chaperones (DnaK/J, GroEL/ES) as the IB-processing factors has been evaluated [2–5], but no effective procedure for the IB elimination has been established yet. In this study, we investigated the role of the AAA + ATPase ClpB in the IB processing in *E. coli* cells. It has been previously shown that ClpB cooperates with DnaK/J and small heat shock proteins IbpA/B in reversing protein aggregation [6]. However, the capability of ClpB to disaggregate bacterial IBs has been questioned [7]. In this work, we employed two aggregation-prone fusion proteins, VP1-β-galactosidase (VP1LAC protein) and VP1-green fluorescent protein (VP1GFP protein) accumulating as IBs in the cytoplasm of *E. coli* cells. These proteins are engineered as fusions with the aggregation-prone VP1 capsid protein of the foot-and-mouth disease virus [8]. The most characteristic feature of these protein aggregates is the co-aggregation of both active and inactive polypeptides [8]. We found that ClpB accumulates in *E. coli* cells upon overproduction of VP1GFP. Importantly, ClpB is essential for resolubilization of IBs formed by the aggregation-prone fusion variants of GFP and β-galactosidase and for recovery of their folded and active conformations. Thus, ClpB may play a key role in the IB processing in *E. coli* cells.

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[1] *Abbreviations used:* IBs, inclusion bodies; VP1- capsid protein of the foot-and-mouth disease virus; VP1GFP, VP1-green fluorescent protein; VP1LAC, VP1-β-galactosidase; wt, wild-type.

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Materials and methods

Bacterial strains, plasmids and growth conditions of bacterial cultures

E. coli strain MC4100 (SG20250) (*ara*D139, Δ(*argF-lac*)*U*169, *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 MC4100Δ*clpB*::kan was supplied by A. Toussaint (Université Libre de Bruxelles, Brussels, Belgium). Plasmids, pJVP1LAC and pTVP1GFP, were kindly provided by Garcia-Fruitos (Institut de Biologia Fonamental and Departament de Genetica and Microbiologia, Universitat Autonoma de Barcelona, Spain). Plasmid pJVP1LAC encodes *E. coli* β-galactosidase fused to the aggregation-prone VP1 capsid protein of the foot-and-mouth disease virus (VP1LAC) [9]. The expression of *VP1LACZ* gene is under the control of tandem λ *pLp*R lytic promoters and repressed by a temperature-sensitive CI857 repressor. Plasmid pTVP1GFP encodes GFP also fused to VP1 capsid protein (VP1GFP) [10]. In this case, the expression of *VP1GFP* gene is under the control of IPTG-inducible p*trc* promoter. Plasmids pClpB95/80, pClpB95, pClpB80 (pGB2-derivatives) carrying an appropriate *clpB* variant together with the σ^{32} – dependent promoter were constructed earlier [11,12]. Plasmid pGB2 (spc^R, str^R) was used as a control [13]. DNA plasmid preparation and transformation of *E. coli* strains were done according to [14].

Bacteria overproducing VP1LAC were grown as described previously [15]. Briefly, bacteria were grown at 28 °C to OD_{600} = ~0.3 and then were transferred to 42 °C to induce the recombinant VP1LAC protein production. To examine the ability of ClpB to process IBs, after a 2 h thermal induction, protein synthesis was inhibited by chloramphenicol (200 μg/ml) and the cultures were further incubated at 28 °C for 3 h. Bacteria overproducing VP1GFP 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} = ~0.4. Then, 1 mM IPTG was added to induce the VP1GFP protein production and the cultures were further incubated at 37 °C for 3 h. Next, protein synthesis was arrested with chloramphenicol (200 μg/ml) and bacteria were transferred back to 28 °C for 3 h to investigate the ability of ClpB to process the GFP IBs. Cultures samples (containing equal amounts of bacteria) collected at different time points after a 3 h incubation at 37 °C and during the period of growth at 28 °C were assayed for GFP fluorescence and separated into the soluble and insoluble protein fractions. To investigate the effect of protein aggregates on the accumulation of ClpB, bacteria overproducing VP1GFP were grown at 30 °C (to exclude a possible heat-shock promoter activation).

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). Detailed procedure for the preparation of the soluble and insoluble protein fractions was described previously [15]. The purified VP1LAC IBs were resuspended in 300 μl of B-PER reagent solution while VP1GFP IBs were resuspended in 300 μl of 10 mM Tris/HCl (pH = 7.4). VP1LAC or VP1GFP in the obtained fractions was detected by measuring the enzyme activity or fluorescence emission, respectively.

Analytical methods

β-Galactosidase activity was determined according to Miller's method [16] and its activity is reported in Miller units ($1000 \times OD_{420}/OD_{600}$ of culture per ml of culture per min of reaction). GFP fluorescence was recorded using a Perkin Elmer LS55 spectrofluorometer with a temperature-controlled cuvette and a magnetically driven stirrer. All measurements were done at 23 °C. Both enzymatic activity and fluorescence were determined in triplicate.

VP1LAC and VP1GFP IBs were analyzed by SDS–PAGE according to [17] and stained with Coomassie Brilliant Blue dye. The stained gels were scanned and analyzed with 1Dscan EX, Scananalytics Inc. Sigma program.

Protein concentration in total cell extracts and in the separated soluble and insoluble fractions was determined by the Bradford method [18] using BSA as a standard.

Western blotting was performed to estimate the amount of ClpB after the IPTG induction of VP1GFP and for identification of ClpB95/80 in VP1GFP IBs. Rabbit polyclonal antisera against GFP 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.

Fluorescence microscopy of bacteria and IBs

E. coli cells overproducing VP1GFP at 37 °C and VP1GFP IBs isolated from these bacteria were observed under Nikon TE-300 inverted fluorescence microscope with 40-fold magnification (the exposure time: 220 ms of transmitted light). Bacteria were collected from the culture and visualized directly in a microscope while purified IBs were previously fixed with 1% agarose in 10 mM Tris (pH = 7.4). The average diameter of purified IBs was measured using the Nikon EZ-C1 3.30 FreeViewer Software and by analysing 30 individual aggregates corresponding to two different fields. The morphology analysis included 150 cells of each tested bacterial strain.

Results and discussion

Overproduction of VP1GFP in E. coli cells induces the accumulation of ClpB

It is known that biosynthesis of heat shock proteins is strongly induced under stressful conditions. Protein aggregation and the formation of IBs may also represent stress enhancing the heat shock protein synthesis [1]. Therefore, we investigated whether the presence of VP1GFP aggregates affects the accumulation of ClpB in *E. coli* cells. Two isoforms of ClpB are produced in *E. coli*: the full length ClpB95 and the N-terminally truncated ClpB80 [19–21]. It has been previously shown that both ClpB isoforms synergistically cooperate during aggregate reactivation [12,15]. Immunoblotting was performed (Fig. 1A) to estimate the ClpB levels in the following *E. coli* lysates: MC4100*clpB+* (control 1), MC4100[pTVP1GFP] and MC4100Δ*clpB*[pTVP1GFP] (control 2). The cultures were analyzed without and with the VP1GFP overexpression, which leads to the IB formation (see Fig. S2). Both ClpB isoforms, ClpB95 and ClpB80 accumulated in the presence of IBs (Fig. 1A). Scanning densitometry of the obtained immunoblot revealed that a 1 h overproduction of VP1GFP at 30 °C resulted in ~3-fold increase in the ClpB95 level (lane 6), as compared to the uninduced control (lane 5) (Fig. 1B). Accurate quantification of the ClpB80 accumulation was not possible due to an apparent non-specific band next to ClpB80 (see Fig. 1A). This increase in the ClpB level is similar to the induction of this chaperone observed when *E. coli* cells were subjected to a mild heat shock (temperature change from 30 to 37 °C or from 37 to 42 °C for 1 h) [22]. After a 2 h induction of VP1GFP, the ClpB level in the cells increased ~4-fold (lanes 7 and 8). This result indicates that the overproduction of an aggregation-prone protein in *E. coli* cells at a non-stressful