Protein aggregation as bacterial inclusion bodies is reversible

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Abstract Inclusion bodies are refractile, intracellular protein aggregates usually observed in bacteria upon targeted gene overexpression. Since their occurrence has a major economical impact in protein production bio-processes, in vitro refolding strategies are under continuous exploration. In this work, we prove spontaneous in vivo release of both β-galactosidase and P22 tailspike polypeptides from inclusion bodies resulting in their almost complete disintegration and in the concomitant appearance of soluble, properly folded native proteins with full biological activity. Since, in particular, the tailspike protein exhibits an unusually slow and complex folding pathway involving deep interdigitation of β-sheet structures, its in vivo refolding indicates that bacterial inclusion body proteins are not collapsed into an irreversible unfolded state. Then, inclusion bodies can be observed as transient deposits of folding-prone polypeptides, resulting from an unbalanced equilibrium between in vivo protein precipitation and refolding that can be actively displaced by arresting protein synthesis. The observation that the formation of big inclusion bodies is reversible in vivo can be also relevant in the context of amyloid diseases, in which deposition of important amounts of aggregated protein initiates the pathogenic process.

Key words: Inclusion body; Protein aggregation; Refolding; Recombinant protein; β-Galactosidase; TSP

1. Introduction

Inclusion bodies (IBs) are major protein aggregates commonly occurring in recombinant bacteria when the expression of plasmid-encoded genes is directed at high rates [1–3]. Despite some physiological factors influencing IB formation have been identified [2], attempts to prevent protein aggregation are in general unsuccessful [3]. Therefore, and since polypeptides embedded in IBs are devoid of any biological activity and therefore usefulness in a biotechnological context, refolding procedures for in vitro protein recovery from purified IBs are under continuous development [4–7]. On the other hand, bacterial IBs are interesting and convenient models for dynamic and structural analysis of protein aggregation, that could serve to better understand biological situations which are difficult to approach experimentally, such as protein deposition in amyloid diseases. Despite this potential interest, bacterial IBs, often viewed as an obstacle in bioproduction processes, have been in general poorly investigated.

In this work, we prove that bacterial IBs are not inert protein aggregates that undergo a parsimonious volumetric grow by product accumulation, but on the contrary, the result of an unbalanced equilibrium between in vivo protein aggregation and solubilisation. Interestingly, this equilibrium can be spontaneously displaced towards protein refolding when protein synthesis is arrested, a situation that conducts to an almost complete IB disintegration and to the appearing of fully active protein forms. This fact can have a significant impact on protein recovery when using bacteria as cell factory. In addition, it offers new data about the general mechanics of protein aggregation and an interesting model to monitor protein aggregation and solubilisation in real time, a possibility that could be relevant also in the context of prionic and other amyloid diseases.

2. Materials and methods

2.1. Bacterial strains, plasmids, proteins and culture conditions

The Lon$^-$ *Escherichia coli* strain BL21 [8] and its LacZ$^-$ derivative BL26 were used for protein production. Plasmid pJVP1LAC is a pJLA602 derivative that encodes a β-galactosidase fusion harbouring the VP1 capsid protein of foot and mouth disease virus (FMDV) [9], whose production is controlled by the lambda p_R and p_L lytic promoters and the temperature-sensitive CI857 repressor. Plasmid pTTSPA is a pTrc99 derivative that encodes a pseudo-wild-type TSP protein (TSPA) with the full biological activities of TSP [10], whose production is under the control of p_{tac} promoter. Production of both VP1LAC and TSPA in *E. coli* results in IB formation. Luria Bertani (LB) medium [8] plus 100 μg/ml ampicillin was used for culture of recombinant cells, and induction of gene expression was achieved by temperature upshift from 28 to 42°C for VP1LAC and by IPTG addition (up to 1 mM) at 37°C for TSPA. At different times after induction of gene expression, protein synthesis was arrested by chloramphenicol addition (up to 200 μg/ml). In TSPA-producing cultures, media were also washed by centrifugation to remove IPTG, immediately prior to chloramphenicol addition. After chloramphenicol addition, pJVP1LAC-carrying cells were incubated at 28°C to allow a proper folding and activity of CI857^{ts} repressor. For some in vivo experiments, the strain JGT17, a *Δibp* derivative of MC4100 [11] was also used.

2.2. IB analysis and purification and determination of protein activity

Procedures for numeric and volumetric IB analysis inside the cells were already described [12], as well as the IB purification protocol by repeated detergent treatment [13]. Since TSP IBs are rather smaller than VP1LAC's, in vivo analyses were done on 24 h- and 3 h-aged IBs respectively. Soluble and insoluble cell fractions were analysed by PAGE and proteins were detected in Western blot by using appropriate sera. β-Galactosidase activity was determined according to Miller's method [14]. TSP activity was determined by plaque counting on confluent cultures of *Salmonella typhimurium* LT2 after incubating cell

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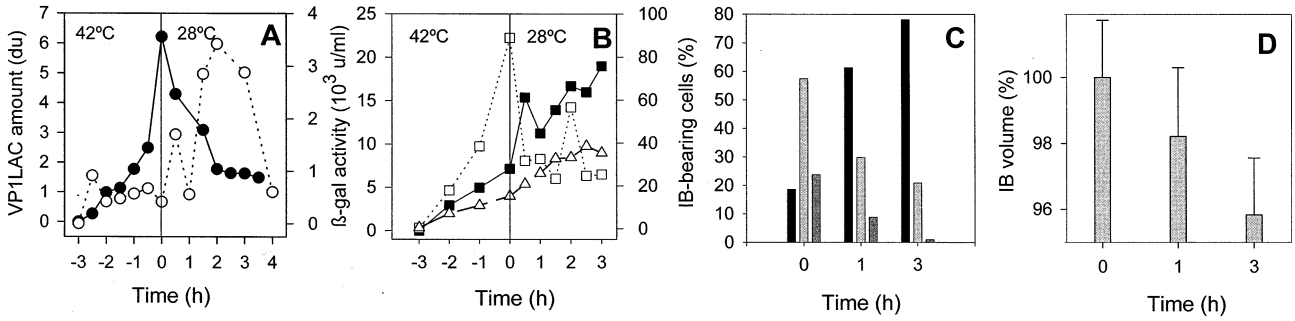

Fig. 1. A: Presence of VP1LAC in the insoluble (black circles, left scale) and soluble (white circles, right scale) cell fractions from BL26 bacterial cultures. The vertical line indicates chloramphenicol addition and temperature downshift. DU are densitometric units. B: β-Galactosidase activity in bacterial cell cultures producing either VP1LAC (black squares, left scale) or CO46 (white squares, right scale). CO46 is a pseudo-wild-type β-galactosidase not forming IBs. The activity in a VP1LAC-producing, IbpAB$^-$ strain is also indicated (white triangles, left scale). U are Miller β-galactosidase units [14]. C: Percentage of cells carrying either none (black bars), 1 (light grey bars), or 2 IBs (dark grey bars), in BL21/pJVP1LAC from the same culture than in A and B. D: Variation in the average volume of remaining IBs. The induction time is indicated in all the panels.

extracts and tailless P22 particles for assembling [10]. When required, cells were disrupted by sonication as described [15]. For in vitro analysis, purified TSP and VP1LAC IBs (10× concentrated) from 3 h-induced cultures, were incubated at about 1.3 μg of protein per ml at 37°C with 20× concentrated, sonic cell extracts from plasmid-free BL26 cells at late exponential growth phase. These cells, were also grown in LB medium at 37°C as indicated above and shifted to 42°C at the middle exponential phase. Protease inhibitors PMSF (to 1 mM) and benzamidine (to 2.5 μg/ml) were also added. As a control, IBs were incubated under the same conditions with the cell-free buffers used for further activity analysis, using Z buffer for VP1LAC IBs [14] and TMBS for TSP IBs [10]. Samples were taken every 30 min for activity analysis as described above, and soluble and insoluble protein fractionation. Proteins in the obtained fractions were analysed by Western blot. In the figures, representative experiments are shown from sets of four replicas.

3. Results

3.1. In vivo refolding of IB protein

In a previous work, we have shown a dynamic transition between soluble and insoluble forms of β-galactosidase fusion proteins in IB-forming cells, that might involve a significant fraction of IB polypeptides and in which proteolysis is connected [16]. Since it had not been still solved whether proteolysis could be a mechanism of IB protein solubilisation or on the contrary, it occurs over already solubilised polypeptides, we have studied β-galactosidase enzymatic activity and IB volumetric evolution after arresting the synthesis of the β-galactosidase fusion protein VP1LAC. Under these conditions, both soluble protein (Fig. 1A) and β-galactosidase activity (Fig. 1B) increase, concomitantly with a reduction in the insoluble VP1LAC fraction (Fig. 1A). Note that in a recombinant β-galactosidase, that does not aggregate as IBs (protein CO46), enzymatic activity is rapidly lost after the arrest of protein synthesis (Fig. 1B) by degradation of the soluble CO46. Moreover, in BL26/pJVP1LAC cells, a simultaneous decrease of IB number and average volume is also observed (Figs. 1C, D, 2, top). Altogether, these results strongly suggest that IB-embedded protein is solubilised in vivo resulting in disintegration of IB particles trough their progressive volumetric reduction. In addition, at least a fraction of IB protein can reach the native conformation with fully biological activity, proving that proteolysis occurs on polypeptides previously released by a proteolytic-independent process. The fact that in an Ibp$^-$ strain, in which IBs are also formed (not shown), β-galactosidase activity is recovered as in the wild-type (Fig. 1B), suggests that IbpA,B proteins are not critical in this refolding process.

In an additional approach to evaluate in vivo IB evolution in a different expression system we monitored IB evolution in cells producing P22 TSP protein IBs. TSP is a homotrimeric protein that undergoes a complex folding pathway from which unfolded intermediates collapse as IBs under overproduction conditions [17,18]. In addition, TSP has been extensively used as a model for the analysis of molecular interactions between aggregated polypeptides [19] and of protein folding and unfolding pathways [20–24]. The arrest of recombinant protein synthesis in IB-carrying cells results, as in the case of β-galactosidase, in IB disintegration (Fig. 2, bottom; Fig. 3D). Loss of TSP in the insoluble cell fraction can be also monitored, as in the case of VP1LAC, by a numeric IB reduction and a concomitant rise of TSP with full biological activity (Fig. 3B). This indicates that the aggregated protein can be released from IBs in absence of protein synthesis and transferred to the soluble cell fraction as TSP monomers, that, also in absence of protein synthesis, can refold as fully active TSP trimers. Note that a rapid increase in the trimeric native form (Fig. 3B) is accounted by an initial lost of the monomeric form from the soluble cell fraction (Fig. 3A) and that a rate transition in the folding pathway between 1 and 2 h is coincident with the appearing of monomeric forms from IB particles, that results again in a further folding rate transition at about 4 h. Some folding intermediates and degradation fragments have also been observed in blots (not shown).

Although it cannot be excluded that IBs formed by other proteins could undergo alternative or less efficient refolding processes, the fact that two structurally different proteins are released with a comparable efficiency from IBs reveal an unexpected context for protein aggregation in bacterial cells, in which IB formation and growth must be regarded as the result of an unbalanced equilibrium between protein synthesis and aggregation. During production of misfolding-prone proteins, bottlenecks in the protein folding assistant network would conduct to deposition of unfolded and misfolded polypeptides, that would be progressively refolded upon chaperone availability, a situation that is strongly favoured when the de novo protein production is arrested.

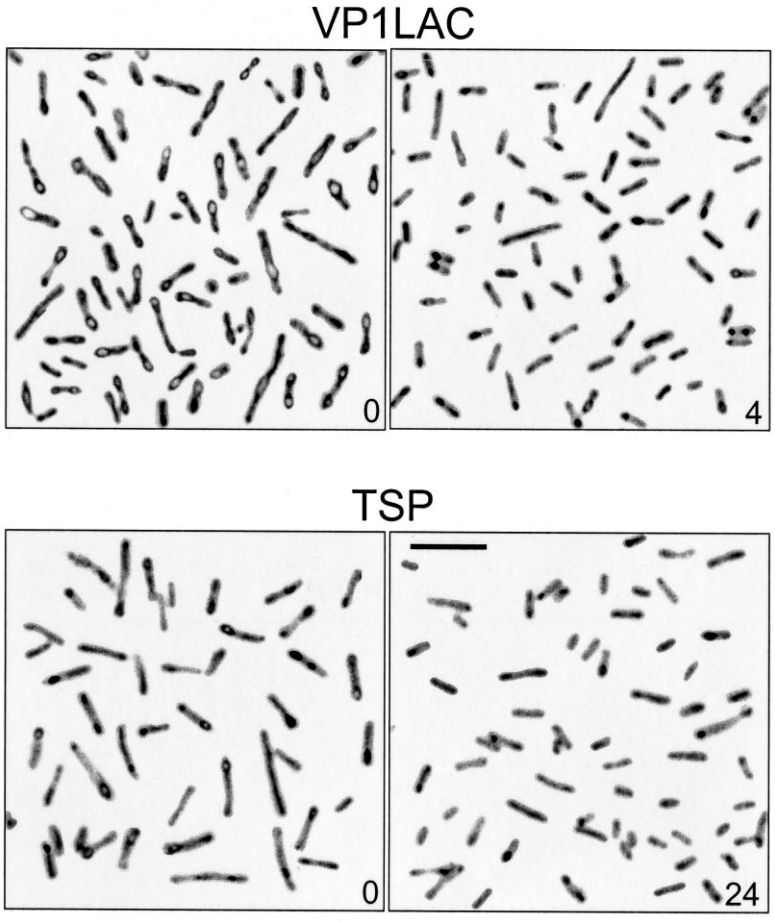

Fig. 2. Micrographies of VP1LAC (top) and TSP (bottom) BL26 producing cells. Numbers indicate time in h after stopping the synthesis of recombinant proteins. The black bar represents 5 μm.

3.2. *In vitro refolding of IB protein*

Some components of the bacterial folding assistant network have been identified, although their sequential implication in the cascade folding process is still under exploration [25–27]. Irrespective of the specific factors acting on bacterial cells on IB protein refolding, the possibility to induce in vitro protein solubilisation from protein aggregates could be of interest in a variety of purposes. In Fig. 4A, we show a rising of a major VP1LAC degradation product (fragment B) [9] and a concomitant increase of β-galactosidase enzymatic activity by incubation of purified IBs in sonic extracts of *E. coli* cells. A simultaneous rising of intact VP1LAC is also observed by high sensitive developing of blots (not shown), but the amount of this species is too low for an accurate determination. β-Galactosidase activity is not detected when IBs are incubated in saline buffer, although in some of these samples, low protein

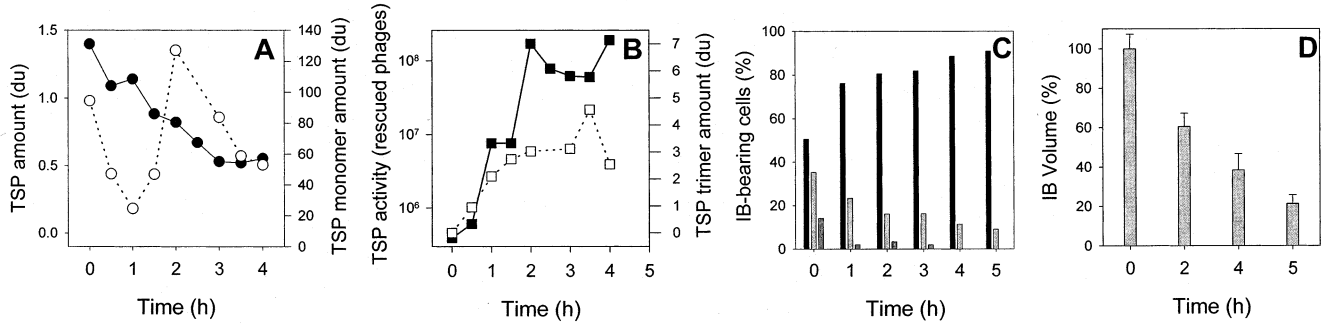

Fig. 3. A: Monitoring of TSP monomer amounts after chloramphenicol addition (time 0) in insoluble (black circles, left scale) and soluble (white circles, right scale) cell fractions. B: TSP activity monitored by phage rescue in the total cell fraction (black squares, left scale) and TSP found in active, trimeric forms in the soluble cell fraction (white squares, right scale). C: Percentage of cells carrying either none (black bars), 1 (light grey bars), or 2 IBs (dark grey bars), in BL26/pTTSPA from the same culture as in A and B. Note that in A, the detection of monomers in the insoluble cell fraction does not necessarily imply that TSP is actually in the monomeric form as embedded into IBs, since for electrophoretic analysis of insoluble protein samples must be denatured before loading. D: Variation in the average volume of remaining IBs. The induction time is indicated in all the panels.

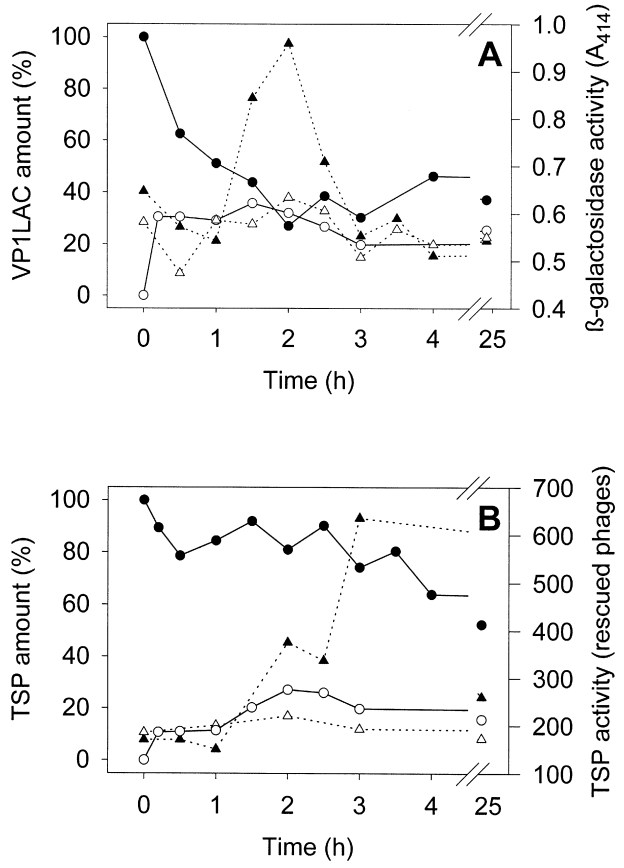

Fig. 4. A: β-Galactosidase activity (right scale) from VP1LAC IBs incubated with crude cell extracts (black triangles) or with saline buffer (white triangles). Protein amounts (left scale) in the insoluble (black circles) and soluble (white circles) cell fractions are also indicated. Whereas the insoluble protein corresponds to the intact VP1LAC, the soluble fraction is mainly composed by a major degradation fragment of 90 kDa [9]. B: TSP activity as monitored by infectious phages rescued from P22 tailless particles (right scale), in TSP IBs incubated with crude cell extracts (black triangles) or with saline buffer (white triangles). Protein amounts (left scale) in the insoluble (black circles) and soluble (white circles) cell fractions are also indicated. Intact TSP monomers are the main component of the soluble cell fraction.

amounts were eventually observed in the soluble cell fraction of some samples (not shown). The intact form of the soluble enzyme in cell extract-treated IB samples is hardly detectable in blots, and since the main degradation fragment is not present in pure IBs [9], this indicates a rapid proteolysis after the release of polypeptides from aggregates, in agreement with the late fading of the enzymatic activity. On the other hand, about 60% of IB protein is lost in about 2 h. In Fig. 4B, an analogous increase of TSP phage rescuing activity is observed in TSP IBs cell extract mixtures, simultaneously to a lost up to 40% of IB protein. Again, the late decrease of TSP activity and concentration of soluble TSP indicates further protein degradation.

4. Discussion

Since early studies on bacterial physiology, IBs were believed to be compact protein aggregates of unfolded polypeptide chains, that being unreachable by proteases and chaperones, remain inert once produced in the cell [1,28]. However, it has been observed enzymatic activity associated to some enzyme-based IBs [29,30] and also the presence of native-like secondary structure in IB protein [31]. In addition, the finding of different conformational states within IB particles [32] has been very recently confirmed by combining scanning electron microscopy and kinetic modelling of in vitro IB tryptic digestion [13]. These observations indicate that as in the case of amyloid fibrils, IBs might be formed by a range of folding intermediates rather that by completely unfolded chains.

Even more intriguing is the observation of a dynamic protein transition between soluble and insoluble cell fractions during volumetric IB growth in vivo [16], that appeared to be linked to the proteolytic digestion. By exploring this event in both a β-galactosidase fusion (VP1LAC) and P22 TSP, we prove here that IB growth is the result of an unbalanced equilibrium between protein precipitation and cell-mediated refolding and solubilisation, that can be spontaneously redirected towards body disintegration by arresting the synthesis of the recombinant polypeptide. Under this situation and when IBs had been already formed, their number and volume rapidly decrease in living cells (Figs 1, 2 and 3), concomitant with a transition of the target protein from the insoluble to the soluble cell fraction and a protein refolding process to gain full functionality. Note that TSP folding is a specially long and complex molecular process [17] to reach deep β-sheet interdigitation between subunits [33]. In the view of these results, it seems reasonable to speculate that components of the bacterial folding assistant network are able to act over aggregates of misfolded protein prompting protein release and correct protein folding from IBs. This solubilisation process is eclipsed when protein synthesis is directed at high rates, but becomes evident when de novo synthesis is arrested. However, note that in both in vivo and in vitro situations, a small protein amount remains insoluble after prolonged experiments, a fact that could be in agreement with the observation of a heterogeneity in the molecular organisation of IB polypeptides as recently described [13]. It is worthy to note, that whereas TSP IBs are homogeneously affected by protein solubilisation (Fig. 3D), recalcitrant species of aggregated VP1LAC seem to be heterogeneously distributed among IBs in the bacterial culture, since a small fraction of these particles displays essentially the same volume in absence of protein synthesis (Fig. 1D). This observation could indicate that different polypeptides can be packaged in different organisation patterns within IB particles.

Independent approaches have proved that chaperones can prevent protein aggregation in different cellular contexts and also favour in vivo solubilisation from aggregates from different origin [34–40]. However, as far as we know, bacterial inclusion bodies have not been explored in this context and its transient nature never reported, despite being extremely common and inconvenient in recombinant bacteria. The data presented here indicate that IB formation is not a dead-end process in the protein quality network [41] of bacterial cells, but a transient situation in which IBs act as a reservoir of misfolded polypeptide chains, over which cell components can still apply its folding assistant potential. The fact that IB solubilisation can also be done in vitro by using cell extracts (Fig. 4), offers intriguing possibilities to solve or diminish one of the main bottlenecks in the use of bacteria as a cell factory for foreign proteins, namely IB for-

mation. More importantly, the fact that protein aggregation can be reverted even from poorly structured elements such as IBs prompts to deeply scrutinise possibilities for therapeutic approaches to amyloid diseases based on the reversion of the aggregation process. This possibility is specially appealing since the recent report that chaperones can act as neurodegenerative repressors [35,36] and modulators of fibril formation [37].

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