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Protein quality in bacterial inclusion bodies

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A common limitation of recombinant protein production in bacteria is the formation of insoluble protein aggregates known as inclusion bodies. The propensity of a given protein to aggregate is unpredictable, and the goal of a properly folded, soluble species has been pursued using four main approaches: modification of the protein sequence; increasing the availability of folding assistant proteins; increasing the performance of the translation machinery; and minimizing physico-chemical conditions favoring conformational stress and aggregation. From a molecular point of view, inclusion bodies are considered to be formed by unspecific hydrophobic interactions between disorderly deposited polypeptides, and are observed as 'molecular dust-balls' in productive cells. However, recent data suggest that these protein aggregates might be a reservoir of alternative conformational states, their formation being no less specific than the acquisition of the native-state structure.

Introduction

Recombinant protein production is an essential tool for the biotechnology industry and also supports expanding areas of basic and biomedical research, including structural genomics and proteomics. Although bacteria still represent a convenient production system, many recombinant polypeptides produced in prokaryotic hosts undergo irregular or incomplete folding processes that usually result in their accumulation as insoluble, and usually refractile, aggregates known as inclusion bodies (IBs) [1,2]. In fact, the solubility of bacterially produced proteins is of major concern in production processes [3,4] because IBs are commonly formed during overexpression of heterologous genes, particularly of mammalian or viral origin. Consequently, many biologically relevant protein species are excluded from the market because they cannot be harvested in the native form at economically convenient yields. Although some recombinant proteins do occur in both the soluble and insoluble cell fractions, many others are only produced as IBs. To date, the solubility of a given gene product has not been anticipated before gene expression. However, it is now clear that the extent of protein aggregation is determined, at least partially, by a combination of process parameters, including culture media composition, growth temperature, production rate (as result of diverse factors, such as gene dosage, promoter strength, mRNA stability and codon usage) [5,6], and the availability of heat-shock chaperones [7,8]. All of these factors can be manipulated to enhance solubility but the operational range is more limited than that required for a competent solubility control. Overexpression of chaperones and other folding modulators along with the recombinant gene has been the most successful approach for the minimization of IB formation. During the past decade, hundreds of articles have described particular chaperone-assisted production experiments with poorly concluding results, often because of inconsistencies when considering different protein species, host cell strains or expression systems [8,9]. Although still a matter of speculation, the origin of such variability might lie in the distinct requirements of different proteins when folding in a prokaryotic environment.

In addition, despite the functional redundancy of the quality control system, the activities of some chaperones (such as DnaK) cannot be completely complemented by others [10], and their titration causes bottlenecks in the folding process [11]. It is also true that an important part of the bacterial protein quality-control system is organized into partially overlapping sequential networks, in which folding intermediates are delivered from one chaperone (or chaperone set) to another [12,13]. This sequential handling would prevent the proper folding of a misfolding-prone species when one crucial folding element is not available at the required concentrations; however, the overexpression of this bottleneck chaperone would make the next step of the folding process limiting.

Alternatively, IBs can be a source of relatively pure protein because they can be easily purified from disrupted cells. By using IBs as a starting material, and after applying *in vitro* refolding procedures, native proteins can be recovered ready for use [14–20]. The main concern about using IBs as a source material for industrial purposes is that *in vitro* refolding procedures are not universal and need to be adapted for each specific protein. In addition, the cost and speed of such refolding procedures are not always convenient in the large-scale formats needed in industry [15,21].

The undesired aggregation of recombinant proteins has been experienced since early recombinant DNA technol-

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structural data that has been collected about IBs during the past five years are now offering the first steps towards an integrated model of protein aggregation in bacteria [22]. In addition, picturing how IB formation is connected to the physiology of the cell during the conformational stress imposed by protein overproduction is now becoming possible.

Morphology and composition

In actively producing recombinant *E. coli* cells, IBs are seen as refractile particles, usually occurring in the cytoplasm [23,24], although secretory proteins can also form IBs in the periplasm [25]. Under electron microscopy, IBs appear rather amorphous [26] but, after detergent-based purification, scanning microscopy reveals them to be rod-shaped particles [24,27]. *In vitro* protease digestion of purified inclusion bodies occurs on IB-associated proteins as a cascade process [28,29] in which target sites are sequentially activated or exposed to the enzyme in a defined manner. This in-order cleavage indicates both conformational flexibility and accessibility of IB proteins. Also, partially digested IBs have a granular architecture [27] that might be compatible with IBs being formed by the clustering of protease-resistant, smaller aggregates. Classical proteomics of IBs showed them to be relatively homogeneous in composition and mainly formed by the recombinant protein itself [30–32]. Although occurring in variable proportions, the recombinant product can reach more than 90% of the total embedded polypeptides [2,22], which is a convenient protein supply for further *in vitro* refolding. The remaining material includes proteolytic fragments of the recombinant protein [33,34], traces of membrane proteins [30,35], phospholipids and nucleic acids [31], at least some of these being contaminants retained during the IB purification procedures [36]. In *E. coli* IBs, the small heat-shock proteins IbpA and IbpB have been identified [22,37,38] in addition to the main chaperones DnaK and GroEL [22,35].

Molecular determinants

The large set of polypeptides forming bacterial IBs are not related, either structurally or sequentially, and include small, large, monomeric, multimeric, prokaryotic or eukaryotic proteins. Thus, aggregation inside bacterial factories has long been considered to be a nonspecific process, resulting in the formation of disordered intracellular precipitates. Accordingly, several general features inherent to the particular molecular status of the protein but irrespective of its nature have been suggested to promote IB formation. These include: high local concentrations of the produced polypeptide; transient accumulation of proteins in totally or partially unfolded conformations, with reduced solubility related to that of the native form [3]; the accumulation of unstructured protein fragments as a result of proteolytic attack [19]; the establishment of wrong interactions with the bacterial folding machinery [39]; the lack of the post-translational modifications needed for the solubility of some eukaryotic

Although such environmental factors are relevant for IB formation, the intrinsic nature of a polypeptide and its sequence also determine its partitioning between the insoluble and soluble cell fractions. Several classical observations, together with recent results, reinforce this view. The high purity of the recombinant protein in IBs, and the recurrent observation that recombinant expression results in the formation of a reduced number of IBs (usually one) [23], suggest that they might be formed by the growth of a small number of initial founder aggregates by a nucleation-like mechanism relying on molecular recognition events. Several observations support this view. First, specificity of polypeptide association during aggregation processes has been seen in *in vitro* refolding studies of proteins in complex protein mixtures [42]. Second, the folding intermediates of different proteins tend to self-associate, *in vitro*, instead of co-aggregating, despite the fact they form IBs when expressed individually in bacteria [43]. Finally, and more interestingly, under certain conditions, co-expression of two proteins from genes carried on the same plasmid results in the formation of two types of cytoplasmic aggregates, each enriched in one type of recombinant protein [44]. This segregation of the protein aggregates is not the result of a temporal dependence of deposition, supporting the view that, seeing as it occurs *in vitro*, aggregation of proteins into IBs is a selective process.

IBs have long been thought to be devoid of all molecular architecture, according to the view that unspecific hydrophobic interactions drive the deposition process. However, pioneering studies in the early 1990s [45–47], together with more recent investigations [48–50], run against this view. The use of attenuated total reflectance infra-red spectroscopy for IBs analysis has shown that, irrespective of the native protein structure, formation of IBs results in the acquisition of significant new β-sheet structures compared with the native conformation, even for β-sheet-rich proteins. The persistence of some native conformation in addition to the presence of disordered chain segments has been also described, the content depending on the particular IB-forming protein [51]. The structural data suggest that the newly formed β-sheet architecture in IBs is stabilized by a network of hydrogen bonds between different chains, resulting in tightly packed, extended intermolecular β-sheets. These β-sheet-rich polypeptides or polypeptide regions would be resistant to proteolysis, and it is enticing to propose that they might constitute the above mentioned multiple protease-resistant nuclei within IBs, whereas proteins or protein segments in native and specially disordered conformations would constitute the protease-sensitive part of IBs.

In this context, an obvious question arises: how do specific interactions that occur during the nucleation process result in a more or less common structure for all IBs? Although only a few studies have addressed this topic for IBs, it has been a key issue in the closely related area of protein misfolding and aggregation into amyloid fibrils. Independent of the forming protein, all amyloid fibrils

by the establishment of non-covalent interactions between polypeptide backbones, which are common to all proteins [53]. For amyloids, it has been proven that the propensities of protein backbones to aggregate are sharply modulated by their amino acid sequences, with certain stretches acting as 'hot spots' from which aggregation can nucleate specifically [54–56]. This can be the case for IBs too. Recently, it has been shown that a preformed IB can act as an effective aggregation seed for the deposition of its partially folded soluble protein counterpart in a dose-dependent manner [49]. Moreover, the seeding process is highly specific because IBs promote the deposition of homologous but not heterologous polypeptides [49]. Sequestering of homologous misfolded species into IBs might be a refined mechanism to reduce the potential toxicity of partially folded monomers or small oligomers [57], of which the solvent-exposed hydrophobic surfaces might interact, improperly, with a large number of cellular components and/or exhaust the *in vivo* folding machinery, thereby hampering the folding and function of the cell proteins. Thus, the establishment of specific interactions during aggregation might be a conserved strategy with a role in cellular protection, which seems to be the case in IB-forming recombinant bacteria [58]. In summary, protein aggregation as bacterial IBs and as amyloid fibrils shows more than one coincident trait (Table 1).

Sequence determinants

The impact of point mutations on IB formation in several protein systems also suggests that the primary structure of a polypeptide somehow determines its propensity to aggregate into IBs, whereby specific changes have a huge impact on solubility. However, to forecast the effect of sequence changes on the aggregation propensity in *E. coli* still constitutes a challenge because the structural and thermodynamic context in which they occur must be taken into account, and these parameters are not easily predictable. Furthermore, consistently identical mutations in different protein systems have been shown to result in dissimilar effects [59–63]. Nevertheless, the increasing number of structural genomic initiatives, and the concomitant need for soluble recombinant proteins, has pushed several attempts to predict IB formation directly from the primary structure [64] but still with inconsistent results. Among the intrinsic factors proposed to be related to the propensity of a polypeptide to be incorporated into IBs are: the size of the polypeptide; its phylogenetic origin; the protein family and/or fold; the charge average; the proportion of aliphatic residues; the *in vivo* half-life; the frequency of occurrence of certain dipeptides and tripeptides within the sequence; the proportion of residues with good β-sheet propensity; and the fraction of turn-forming residues. The reasons behind the discordance among approaches rely on the inherent difficulty of the addressed problem, namely aggregation propensity is the net result of several extrinsic and intrinsic factors and many of them are important to different extents depending on the protein and expression contexts [65]. In addition, it is clear that the solubility of recombinant heterologous proteins has nothing to do with the forces that have shaped sequences during evolution. Thus, it is implausible that particular polypeptide properties, which lead to increased solubility of a recombinant protein, would dominate in any given group of proteins. This hampers the detection of relevant patterns influencing IB formation.

Table 1. Main functional and structural traits of bacterial inclusion bodies resembling those of amyloids

Feature	Refs
High purity of the aggregate	[23]
Aggregation mainly from folding intermediates	[49,89]
Sequence-specific aggregation	[43,49]
Chaperon-modulated aggregation	[11,90]
Seeding-driven aggregation	[49]
Aggregation propensities strongly affected by point mutations	[91–95]
Reduced aggregation by stabilization of the native structure	[96,97]
Intermolecular, cross β-sheet organization or in general, enrichment of β structure	[47,49]
Fibril-like organization (of soluble protein aggregates)	[86]
Amyloid-tropic dye binding	[49]
Enhanced proteolytic resistance (of a fraction of IB	[27,28]

Protein quality and dynamics

Overall, recent data suggests that IBs might embrace conformational states different to those observed in the soluble cell fraction, ranging from enriched β-forms to native or native-like structures [45,48–50] (Figure 1). The heterogeneous conformational status of IB protein was hinted by the modeling of *in vitro* IB proteolytic digestion, where different species with distinctive proteolytic sensitivity were detected [27,28]. Such heterogeneity is probably supported by the fact that the volumetric IB growth during gene overexpression is the result of unbalanced protein deposition and simultaneous cell-driven physiological removal. Interestingly, at least a fraction of IB protein is in continuous dynamic transition between soluble and insoluble cell fractions [33] and, in the absence of protein synthesis, cytoplasmic IBs are almost completely disintegrated in a few hours [66]. Therefore, rather than being mere molecular 'dust-balls' of the folding machinery, IBs are protein reservoirs that are profoundly integrated in the protein quality system of the cell [22], and the embedded protein is under continuous quality surveillance. Disaggregating ATPase-associated chaperones (AAA^+), sharing conserved ATP binding and hydrolysis motifs (essentially ClpB), are probably key elements in IB protein release because they are responsible for protein reactivation in thermally stressed cells [67–70]. Small heat-shock proteins (IbpAB), commonly associated with IB proteins [38,71], are also important contributors to the disintegration process, acting in a chaperone team that includes ClpB and DnaK [72,73]. Other cytoplasmic chaperones, such as GroEL, GroES and ClpA, are probably assisting removal of the IB protein because, upon arrest of protein production, IBs are more stable in their respective absence

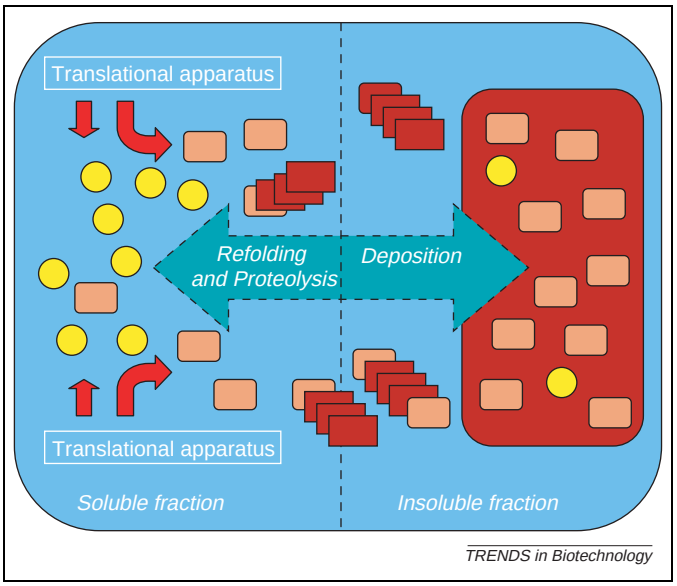

Figure 1. Recombinant proteins produced in distant translational factories within the bacterial cytoplasm occur in either soluble or insoluble cell fractions. Such entities are virtual cell compartments (indicated by a vertical dashed line) between which proteins are distributed according to their fractionation under high-speed centrifugation. A fraction of *de novo* synthesized polypeptides can immediately reach the native conformation and are fully functional (yellow spheres). Other molecules enter into incorrect, dead-end folding pathways, are non functional and tend to aggregate because of the presence of solvent-exposed hydrophobic surfaces (small brown boxes). Aberrant folding forms and folding intermediates can have properly folded domains that, if embracing active sites, might be still fully or partially functional, although tending to aggregate (orange boxes). The backbones of these protein forms can interact in a sequence-dependent manner and under second-order kinetics to form small, β-sheet-enriched, soluble aggregates, organized as fibers or other cluster types. Soluble aggregates are trapped, specifically, in larger aggregation nuclei, forming one or a few IBs (vertical brown box in the insoluble cell fraction) according to first-order kinetics. Therefore, IBs contain both inactive (unfolded) and active (partially folded or eventually properly folded) protein species that might self-organize in a concentric manner. Here, native-like species surround unfolded, densely packaged and proteolytically stable polypeptide chains. Protein material is steadily transferred between these virtual cell compartments by either deposition into IBs or refolding and/or proteolysis of IB proteins, generating a conformational continuum between soluble and insoluble cell fractions. Therefore, incorrect folding and aggregation, or proper folding and solubility, are not perfectly pair-matched events because both active and inactive protein forms can be found in either the soluble or the insoluble fractions.

components [22,35,38]. Intriguingly, most cellular DnaK molecules have been observed at the IB interface [26], where this chaperone probably acts by refolding or releasing IB polypeptides in cooperation with ClpB and IbpAB [67,72,74,75]. Recent insights on the disaggregation process have provided fascinating details about its molecular mechanics. The protein ClpB recognizes substrates through the conserved Tyr251 residue sited at the central pore of the first AAA domain. This fact suggests a translocation event for ClpB-mediated protein removal [76,77] that acts on discrete protein molecules rather than on aggregated sections [78]. Both DnaK and ClpB middle domains might also contribute by providing an unfolding force in a still unsolved mechanism, acting in coordination with the translocation event [79].

Conversely, it seems that proteases are secondary tools for aggregate processing, acting on IB polypeptides once released [66] or during disaggregation [80]; however, *in situ* digestion of IB protein has been suspected, through indirect *in vivo* and *in vitro* observations [23,28,80,81]. In support of a direct proteolytic attack, the absence of either Lon or ClpP proteases largely minimizes IB disintegration [82]. However, in a $ClpP^{-}$ background, IB proteins released to the soluble cell fraction remain stable and can refold to a functional form [82], highlighting this enzyme as a controller of the quality of disaggregated proteins.

The heterogeneous conformational nature of IB proteins is, in addition, reflected by the relatively high activity of IBs formed by enzymes such as galactosidases and other glucanases [6,10,83] (Table 2). Recently, the same has been observed for aggregating fluorescent proteins that generate highly emitting IBs [84]. In fact, when analyzing the specific activity of soluble and IB forms of β-galactosidase fusions, such values are within the same order of magnitude [10]. This similarity can be partially attributed to the occurrence of 'soluble aggregates' [85], namely clusters of soluble but biologically inactive protein, organized as fibers, which might eventually be among IB precursors [86]. Such elements would

Table 2. Some structural and functional evidence that properly folded protein species are a significant component of bacterial IBs

IB protein	Structure (determination method)	Biological activity (% relative to the soluble counterpart, when determined)	Refs
Green- and blue-fluorescent protein fusions		High IB fluorescence emission *in* vivo (between 20 and 30%)	[84]
β-galactosidase and β-galactosidase fusion proteins		High specific activity in purified IBs (from around 30 up to more than 100%)	[6,10,84]
Di-hydropholate reductase		Low activity in purified IBs (6%)	[84]
Endoglucanase D		High activity in purified IBs (25%)	[83]
β-lactamase		Detectable activity in purified IBs	[87]
HtrA1 serine protease		Detectable activity in purified IBs	[87]
Interleukin-1 β	Native-like secondary structure (FTIR)[a]		[45]
Several α-helix-rich hyperthermophilic proteins	Native-like secondary structure (FTIR; NMR; CD)[b,c]		[98]
TEM β-lactamase	Native-like secondary structure (FTIR)		[47]
Lipase	Native-like secondary structure (FTIR)		[50]
Human granulocyte-colony stimulating factor	Native-like secondary structure (FTIR)		[99]
Human growth hormone	Native-like secondary structure (FTIR)		[100]
Human interferon α 2b	Native-like secondary structure (FTIR)		[100]

[a]FTIR, Fourier transformed infrared spectroscopy

reduce the average specific activity of the recombinant enzyme in the soluble cell fraction. Contrarily, an important part of the IB protein population must be properly folded and coexist with the background intermolecular β-sheet organization [49] (Figure 1). Again, this might be indicative of conformational variability within IBs as a result of either native-like and β-enriched polypeptides, polypeptides trapped by β-enriched aggregation determinants (but keeping properly folded active site domains), or a combination of both. Although the specific activity of IB enzymes relative to their soluble versions is highly variable when comparing different proteins (Table 2), IBs formed by enzymes seem to be immediately useful in bioprocesses; they can skip any refolding step because their porous nature would permit substrate processing by the active enzyme molecules [84]. Importantly, the availability of IbpAB and its occurrence in enzyme IBs significantly enhances their biological activities [87]. This observation confirms that these small heat-shock proteins, believed to preserve the folding-competent state of target proteins [88] and keep them suitable for refolding [67,72], are also efficient at preserving their native structure within aggregates.

Conclusions and future prospects

Rather than being 'scrambled eggs', bacterial inclusion bodies are dynamic and conformationally diverse structures, formed by a sequence-selective aggregation process that is probably driven by certain 'hot spots' within the protein sequence. Furthermore, neither are they the dead-end of deficient folding processes but rather the transient reservoirs of aggregated polypeptides that are still under the quality control surveillance of cell chaperones and proteases. Recent insights into IB structure reveal that native or native-like proteins, or protein domains, coexist with β-sheet-rich intermolecular assemblies that share functional and architectural features with amyloid aggregates. In addition, the biological activity of enzymes and fluorescent proteins forming IBs is not dramatically lower than their soluble counterparts. Deeper exploration of this fact will open intriguing possibilities for the biotechnological industry.

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