Fine architecture of bacterial inclusion bodies

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Abstract The molecular organisation of protein aggregates, formed under physiological conditions, has been explored by in vitro trypsin treatment and electron microscopy analysis of bacterially produced inclusion bodies (IBs). The kinetic modelling of protein digestion has revealed variable proteolysis rates during protease exposure that are not compatible with a surface-restricted erosion of body particles but with a hyper-surfaced disintegration by selective enzymatic attack. In addition, differently resistant species of the IB proteins coexist within the particles, with half-lives that differ among them up to 50-fold. During in vivo protein incorporation throughout IB growth, a progressive increase of proteolytic resistance in all these species is observed, indicative of folding transitions and dynamic reorganisations of the body structure. Both the heterogeneity of the folding state and the time-dependent folding transitions undergone by the aggregated polypeptides indicate that IBs are not mere deposits of collapsed, inert molecules but plastic reservoirs of misfolded proteins that would allow, at least up to a certain extent, their in vivo recovery and transference to the soluble cell fraction.

Key words: β-Galactosidase; Protein folding; Aggregation; Proteolysis; Recombinant protein

1. Introduction

Chaperones and proteases are major components of the cellular protein quality control system devoted to prevent the occurrence of misfolded polypeptides. In mammalian cells, the proteolytic escape of incorrectly folded proteins promotes their irreversible accumulation as insoluble amyloid plaques and the consequent development of severe degenerative diseases of growing impact [1]. Different protein species are involved in such diseases. However, a common, defined fibril structure is observed upon aggregation [2] irrespective of the primary sequence and the native folding pattern [2–5].

On the other hand, inclusion bodies (IBs) are refractile aggregates of misfolded, insoluble protein commonly observed upon targeted gene overexpression in bacterial cells [6]. IBs are not homogeneous in protein composition. Many cell folding-assistant proteins and proteolytic fragments of the main polypeptide component are found embedded as variable fractions of the total protein content [7–9]. Despite this heterogeneity, transmission electron microscopy has often pictured IBs as amorphous aggregates lacking defined structural organisation [10]. Here, by combining scanning electron microscopy and a kinetic modelling of IB protein digestion during trypsin treatment, we have observed unexpected architectural features of IBs and the coexistence of distinct populations of aggregated protein with different conformational states. These intriguing results prove a particular organisation of IB proteins that, at difference of the mammalian counterpart aggregates, can explain the in vivo dynamic transition between aggregated and soluble polypeptides recently observed in IB-forming bacteria [11].

2. Materials and methods

2.1. Bacterial strain, plasmids and culture conditions

The *Escherichia coli* strain BL21 [12] was used as IB factory, since the absence of protease La stimulates IB formation [13]. Plasmids pJLACVP1 and pJVP1LAC [13,14] encode two closely related hybrid proteins in which the foot and mouth disease virus VP1 capsid protein is fused to either the carboxy- or amino-termini of *E. coli* β-galactosidase, respectively. In these vectors, transcription of the encoding genes is directed by the lambda p_R and p_L lytic promoters and controlled by the temperature-sensitive CI857 repressor. Luria–Bertani medium [12] plus 100 μg/ml ampicillin was used for the culture of recombinant bacteria. Cultures were first grown at 28°C and 250 rpm. At the beginning of the exponential phase (around 0.4 OD_{550} units), flasks were transferred to a pre-warmed bath at 42°C and further incubated at 250 rpm at this temperature up to 24 h.

2.2. IB sampling and purification

Culture samples of 1.5 ml were periodically taken during the production phase, at 1, 3, 5 and 24 h after temperature shift, and IBs were purified by repeated detergent treatment as follows. Cells were harvested by centrifugation at 12 000×*g* (at 4°C) for 15 min and resuspended in 200 μl of lysis buffer (50 mM Tris–Cl pH 8, 1 mM EDTA, 100 mM NaCl), plus 30 μl of 100 mM protease inhibitor PMSF and 6 μl of 10 mg/ml lysozyme. After 30 min of incubation at 37°C under gentle agitation, NP-40 was added at 1% (v/v) and the mixture incubated at 4°C for 30 min. Then, 3 μl of DNase I (from a 1 mg/ml stock) and 3 μl of 1 M $MgSO_4$ were added and the resulting mixture was further incubated at 37°C for 30 min. Protein aggregates were separated by centrifugation at 12 000×*g* for 15 min at 4°C. Finally, IBs were washed once with the same buffer containing 0.5% Triton X-100 and once with sterile PBS. After a final centrifugation at 12 000×*g* for 15 min, pellets were stored at −80°C until analysis.

2.3. Trypsin treatment and electrophoretic analysis of IBs

Purified IBs containing around 15 ng/μl of recombinant protein were resuspended in 300 μl of Z buffer (0.06 M Na_2HPO_4, 0.04 M $NaH_2PO_4{\cdot}H_2O$, 0.02 M KCl, $MgSO_4{\cdot}7H_2O$, pH 7.0) and incubated with trypsin (to 100 μg/ml) at 37°C. During 24 h incubation, samples were periodically withdrawn and soybean trypsin inhibitor (to 200 μg/ml) was added to stop the digestion. Afterwards, samples were stored at −20°C until Western blot analysis. Simultaneously, negative controls were performed with the same samples without adding trypsin.

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Trypsin-treated IBs were boiled for 10 min in denaturing buffer (125 mM Tris–Cl pH 6.8, 6% SDS, 20% glycerol, 10% β-mercaptoethanol) plus 8 M urea and loaded on 3.5% stacking and 7% polyacrylamide gel electrophoresis separation gels. Protein bands were detected by immunoblot, using anti-β-galactosidase polyclonal serum. Protein amounts were estimated by measuring band areas with one-dimension Intelligent Quantifier® software after high resolution scanning.

2.4. Electronic microscopy

Purified IBs were fixed with 2.5% glutaraldehyde in phosphate buffer (Na_2HPO_4 0.9 M, $Na_2H_2PO_4$ 0.06 M, pH 8.0) for 1 h at 4°C. To remove the cross-linker agent, samples were retained on a nucleopore membrane and washed twice with the same buffer. Afterwards, they were dehydrated by consecutive, 5 min washing steps with increasing concentrations of ethanol in water (30, 50, 70, 90 and 100%). Ethanol was finally evaporated by reaching the critical point. Finally, membranes were covered with a 100 nm layer gold for a further observation under a scanning microscope Hitachi S-570.

2.5. Modelling of IB protein digestion

Proteolysis of IB intact recombinant proteins was modelled as follows. Being $N(t)$ the non-fragmented protein (in absolute densitometric units) at time t, digestion of IB protein occurs experimentally according to the following non-linear differential equation:

$$N'(t)/N(t) = -qN(t)^{p-1};\ p>1,\ q>0 \quad (1)$$

For heterogeneous IBs containing more than one protein species, namely n, composition is described as follows:

$$N(t) = \sum_{i=1}^{n} N_i e^{-q_i t} \quad (2)$$

The specific parameters for each protein species are q_i, while the fractions $N_i/N(0)$ represent the mixing proportions. The function described in Eq. 2 satisfies an n-order linear homogeneous differential equation with constant coefficients, such as

$$f(N, N', ..., N^{(n)}; q_1, q_2, ..., q_n) = 0 \quad (3)$$

From numerical evaluations of $N,N',...,N^{(n)}$ in intermediate degradation times, a linear over-determinate system of equations was built in order to estimate the parameters q_i in Eq. 3. Details of this procedure will be described elsewhere (Cubarsi et al., in preparation). The numerical estimation of the derivatives directly from experimental data led to non-consistent results. For this reason, the derivatives in Eq. 3 were explicitly evaluated from Eq. 1. This clearly produced a smoother and better determination of the derivatives. Thus, Eq. 3 was converted into the following form

$$g(N, p, q; q_1, ..., q_n) = 0 \quad (4)$$

Finally, the mixing proportions were obtained by solving another linear over-determinate system of equations, associated with Eq. 2, for the complete set of experimental data.

3. Results

3.1. Proteolytic stability of IB proteins

Isolated IBs, built up by either VP1LAC or LACVP1 β-galactosidase fusion proteins, were submitted to in vitro proteolytic digestion by incubation with trypsin. For this analysis, we used bodies obtained from cells in which recombinant gene expression had been induced for different periods forming differently aged IBs, spanning from 1 to 24 h. During trypsin treatment, loss of the intact fusion species was observed in all the cases (see one example in Fig. 1). This was concomitant with a transient increase of anti-β-galactosidase immunoreactive degradation fragments, derived from a proteolytic cascade, that is comparable to that observed in vivo [15]. By plotting an intact protein amount versus time, a non-constant degradation velocity was revealed, being higher during the first minutes but rapidly declining with time (Fig. 2). Previous proteolysis assays performed under the same conditions [16] did not suggest any enzyme limitation responsible for variation in the proteolysis rate. In addition, proteolysis of the degradation intermediates was completed after 6 h (Fig. 1) under nearly constant rates (not shown), indicative of a peculiar protease susceptibility of the full-length forms of the proteins.

Half-lives of these intact length forms were roughly approached as ln 2/b by adjusting a simple exponential low

$$N(t) = N(0)e^{-bt} \quad (5)$$

and the obtained values are depicted in Fig. 2. For both proteins, protease resistance increased with the age of the containing bodies. Interestingly, while this observed variation is rather constant for LACVP1, it is rapidly stabilised in VP1LAC. Surface digestion has reasonably been suggested for IB protein proteolysis under in vitro protease exposure [10], and consequently, IB topology could eventually influence digestion rate. However, for a surface-restricted IB erosion, the dramatic difference in IB volume found when comparing LACVP1 and VP1LAC IBs [17] would generate greater discrepancies in the observed half-lives. In addition, the volumetric growth of VP1LAC IBs continues up to 24 h, as it also occurs for LACVP1 IBs [11], proving that IB volume is not a key factor in determining the degradation kinetics.

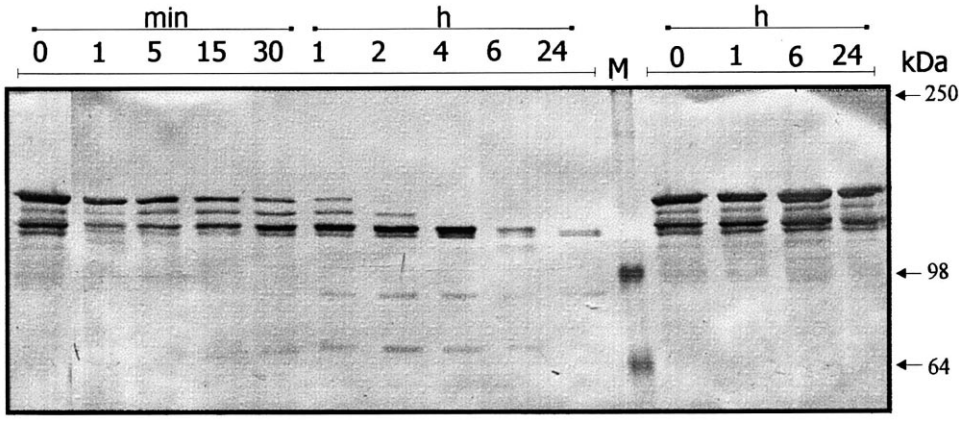

Fig. 1. Western blot of trypsin-treated LACVP1 IBs (aged 5 h) at different incubation times (expressed in min and h). The upper band corresponds to the full-length LACVP1. At the right side, IBs incubated under the same conditions without protease. Lane M indicates molecular weight markers whose apparent masses are indicated by arrows.

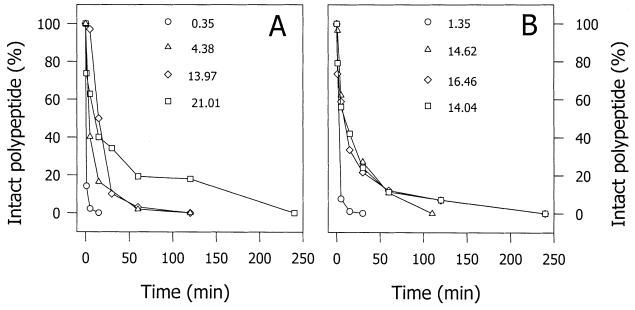

Fig. 2. Evolution of intact IB fusion proteins (LACVP1 in A, VP1LAC in B) during trypsin exposure. Differently aged bodies were processed and are indicated as follows: 1 h, circles; 3 h, triangles; 5 h, diamonds; 24 h, squares. Half-lives estimated from Eq. 1 (in min) are also indicated for each experiment.

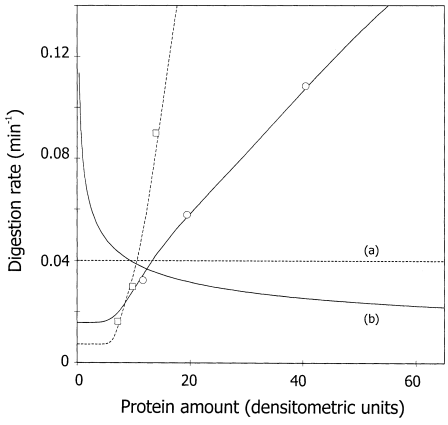

Fig. 3. Digestion rates of IB protein versus protein amount obtained from a three point estimation of 5 h-aged bodies for VP1LAC (circles) and LACVP1 (squares). In addition, expected rates are plotted according to different models of IB digestion, namely constant rate (a, dashed line), surface-restricted erosion (b, continuous line) and mixture of protein species with constant but distinguishable digestion rates, as modelled from the experimental data for both VP1LAC (continuous plot) and LACVP1 (dashed plot). Note that data at zero protein value indicate the digestion rate for the most protease-resistant protein form found in each type of IB, and it is the inverse of the half-lives of fraction 3 as indicated in Table 1. This resistant form is the unique remaining in extensively digested IB particles.

3.2. Heterogeneity of IB protein stability

The results presented above suggest that factors other than IB topology, like intrinsic protein stability or molecular organisation within IBs, would be main determinants of IB digestion dynamics. Therefore, to investigate in more detail the mechanics of protein digestion and the putative influence of body volume, digestion experiments were repeated for 5 h-aged VP1LAC IBs and 3, 5 and 24 h-aged LACVP1 bodies. Additional sampling of LACVP1 IBs was of particular interest because of the time-dependent variation of half-life observed in LACVP1-built bodies as discussed above (Fig. 2). With this set of duplicated data, evolution of intact protein was modelled according to Eq. 1, and the relevant parameter p was obtained for each individual experiment (Table 1). In principle, a Poisson stochastic process [18] would be expected to account for the digestion kinetics. Then, the probability density function for the time interval from trypsin addition up to the first molecular proteolytic event would be given by Eq. 1, rendering $p = 1$.

However, such behaviour would imply a constant digestion rate (Fig. 3, plot a), which is obviously inconsistent with the experimental data (Figs. 2 and 3). An alternative possibility of a concentric layer erosion (a surface-restricted proteolytic attack of IB protein) also predicts a digestion profile that does not fit with the experimentally obtained kinetics (Fig. 3, plot b). Also, note that in this case, p would be 2/3 and that the approximated p values for all the performed experiments are indeed higher than one (Table 1).

Therefore, a more plausible hypothesis can be given by assuming a heterogeneous nature of the IB protein in which more than one different protein species (1, 2, 3 and so on, with increasing proteolytic susceptibility) would coexist. According to this model, the composition of IBs is accurately described by Eq. 2. Under protease exposure, each of these species follows an individual Poisson fragmentation process resulting in distinguishable half-lives, whose combination would render the average half-lives indicated in Fig. 2. While a two-component system explains qualitatively the degradation kinetics, for most of the experiments, the minimal number of protein species required to actually account for the observed data is three (Table 1).

As predicted, these protein species exhibit distinguishable proteolytic sensitivity and they occur at different molar proportions (Table 1), being data from duplicated experiments highly consistent. Except experiment 1 of 5 h-aged LACVP1 IBs, in which only two protein forms could explain numerically the degradation kinetics, a third fraction of a highly sensitive form (half-life between 1 and 6.5 min) is observed

Table 1
Protein composition and stability in differently aged IBs

Protein	IB age (h)	Experiment number	p	Protein species					
				1		2		3	
				Half-life (min)	Content (%)	Half-life (min)	Content (%)	Half-life (min)	Content (%)
VP1LAC	5	1	1.91 ± 0.11	1	1.5	3.5	65.5	26.5	33
VP1LAC	5	2	1.62 ± 0.06	3.5	8	11	68	77	24
LACVP1	3	1	2.46 ± 0.37	1	27	4	27	50.5	46
LACVP1	3	2	1.72 ± 0.22	1	24	4.5	23	22.5	53
LACVP1	5	1	3.74 ± 1.03	nd	nd	2.7	58	64	42
LACVP1	5	2	1.88 ± 0.32	1.6	34	6	15	43	51
LACVP1	24	1	1.81 ± 0.63	4	19	10.5	42	75.5	39
LACVP1	24	2	1.55 ± 0.31	6.5	18	19.5	56	103.5	26

nd, not detected.

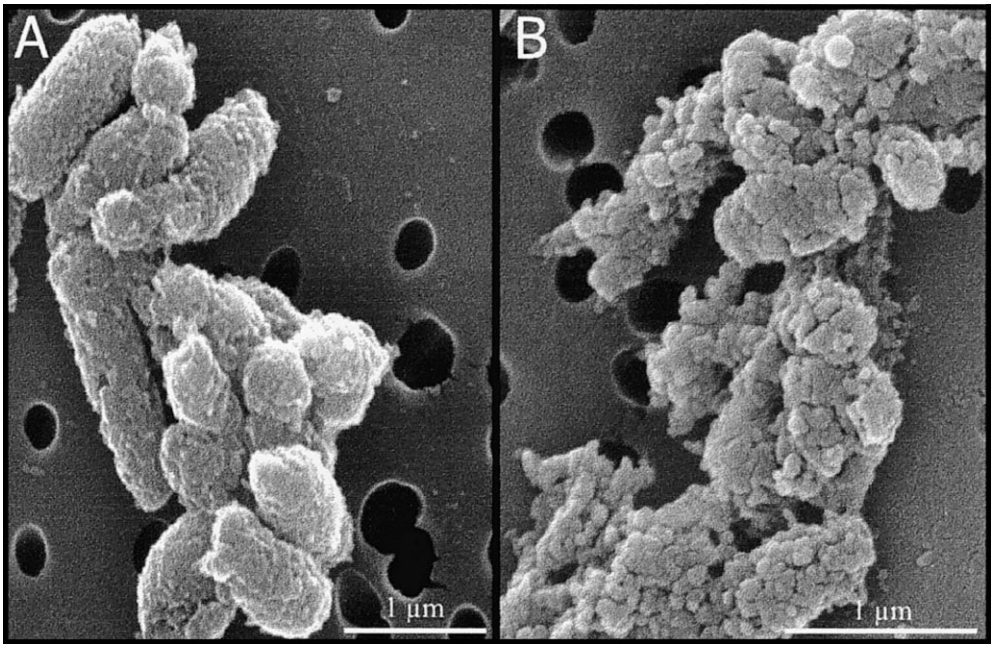

Fig. 4. Scanning electron micrographs of purified VP1LAC (aged 5 h) before (A) and after 5 min of trypsin treatment (B). Similar images were obtained with the smaller LACVP1 IBs (not shown). These pictures are representative of a large number of examinations.

in all cases, abounding more in LACVP1 (from 18 to 34%) than in VP1LAC (less than 8%). The main component of VP1LAC bodies is an intermediate stable protein form (about 68% of total IB protein), while a highly stable species accounts for between 24 and 33% of protein. Interestingly, the half-life of the more stable fraction increases dramatically, although its proportion in the IB protein pool remains nearly constant or even declines. This age-dependent enhancement of fraction 3 stability (between 0.5- and 5-fold) could be a main contributor of the progressively increasing trypsin resistance observed concomitantly with the age of LACVP1 IBs (Fig. 2). Lesser but still detectable stabilisation of fractions 1 and 2 could also be relevant.

3.3. Architectural analysis of digested IB

The model presented above explains successfully the dynamics of IB protein degradation by the presence of at least three protein species that are digested at different rates (Table 1). However, it does not provide information about if these fractions co-locate in heterogeneous IBs or, alternatively, they segregate into different body particles. To approach experimentally this question, purified IBs were examined under SEM during trypsin treatment. While non-treated bodies exhibited a rood-shaped morphology with a smooth surface (Fig. 4A), protease exposure generates profound fragmentation lines that sub-particulate IBs and released sub-body spherical and rood-shaped pieces (Fig. 4B) that further disappear at longer incubation times (not shown). This observation confirmed that the proteolytic attack is not surface-restricted and indicated that protease activity has a selective impact on IB solvent-exposed surface. In addition, the obtained images are compatible with a heterogeneous protein composition, regarding proteolytic stability, that is not dependent on surface accessibility to proteases. Interestingly, this fragmentation pattern is seen in the vast majority of the observed particles, suggesting that IBs built of homogeneously resistant polypeptides, if they occur, are rare among IB-producing bacteria.

4. Discussion

Although poorly studied at a molecular level, bacterial IBs are believed to be inert, unorganised aggregates of completely protein that protect the embodied polypeptides from in vivo proteolytic attack [6]. However, a number of independent observations seriously challenge this assumption. Firstly, under certain conditions, enzymatic activity is found associated to enzyme-based IB particles even after stringent washing [19,20]. Since the specific activities ranged from one-third [19] to almost the same values [20] than that of soluble enzyme, the presence of correctly folded polypeptides in IBs, rather than a mere contamination, should be considered. This is in agreement with the finding of native-like secondary structure in IB protein [21] and the suggestion of different protein conformations within bodies [10], that are greatly influenced by chemical and physical parameters during IB building [22].

On the other hand, unexpectedly short in vivo half-lives have been described for both loose aggregated [15] and true IB protein [23], being even lower than those of the soluble counterparts. In this line, protein release and degradation from body-like proteinaceous structures had already been reported in early studies [24,25]. Very recently, we have observed extremely fast protein release from cytoplasmic IBs of β-galactosidase fusion proteins [11] and other recombinant proteins (Carrió et al., manuscript in preparation), during volumetric IB growth in living cells [17]. This finding proves an important dynamic transition between soluble and insoluble polypeptides that would be in agreement with a more flexible model of the IB structure.

In this context, results presented here strongly suggest that more than one single protein species with distinct protease susceptibility coexist (Table 1), thus accounting for a declining

degradation rate of IB protein (Figs. 2 and 3). Since in β-galactosidase, like in β-lactamase [10] and likely in most of the proteins, protease sensitivity depends on protein conformation [26,27], different folding states of IB protein must be present in LACVP1 and VP1LAC IBs. In addition, a time-dependent increase of half-lives in all the protein fractions during body building (Table 1) is indicative of structural transitions occurring within LACVP1 IBs during protein accumulation. Since degradation does not occur through surface erosion (Figs. 3 and 4), differential protease sensitivity cannot only be explained by substrate exposure, and determinants more complex than the simple surface topology would be actually modulating the digestion dynamics. The relevant percentage of highly sensitive protein (Table 1) and the appearing of deep fragmentation lines (Fig. 4) suggest an invasive trypsin activity selectively attacking defined protein sectors. Apart from the heterogeneous IB protein composition itself, protease invasiveness could be favoured by the high level of IB voidage previously revealed by density analysis [28]. Accordingly, parameter p from Eq. 1 can be observed as a numerical indicator of the IB erosion topology. For values close to 2/3, it would indicate surface erosion, close to 1, homogeneous protease diffusion within single species bodies, and for p values around 2, what is in fact the actual case (Table 1), a polarised protease resistance generating complex, hyper-surfaced IB structures during body disintegration that permit efficient enzyme penetration. The release of protease-resistant pseudo-spherical particles (Fig. 4) and the mutually exclusive pattern of IB growth in the bacterial cytoplasm [17] could also be in agreement with an IB construction process in which pre-aggregated protein particles with protease-resistant cores combine to form one unique higher order body element per cell.

The data presented here, together with other published side observations, show IBs as highly dynamic, flexible and self-organised proteinaceous structures formed by heterogeneously misfolded polypeptides, that support both inner protein folding transitions but also an active protein exchange with the soluble cell fraction.

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