Effects of recombinant protein misfolding and aggregation on bacterial membranes

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ABSTRACT

The expression of recombinant proteins is known to induce a metabolic rearrangement in the host cell. We used aggregation-sensitive model systems to study the effects elicited in *Escherichia coli* cells by the aggregation of recombinant glutathione-S-transferase and its fusion with the green fluorescent protein that, according to the expression conditions, accumulate intracellularly as soluble protein, or soluble and insoluble aggregates. We show that the folding state of the recombinant protein and the complexity of the intracellular aggregates critically affect the cell response. Specifically, protein misfolding and aggregation induce changes in specific host proteins involved in lipid metabolism and oxidative stress, a reduction in the membrane permeability, as well as a rearrangement of its lipid composition. The temporal evolution of the host cell response and that of the aggregation process pointed out that the misfolded protein and soluble aggregates are responsible for the membrane modifications and the changes in the host protein levels. Interestingly, native recombinant protein and large insoluble aggregates do not seem to activate stress markers and membrane rearrangements.

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1. Introduction

Environmental modifications can alter the cell physiological conditions and induce protein unfolding and aggregation in both bacterial and eukaryotic cells. The presence of aggregates is promptly sensed by cells and triggers specific responses to counteract protein misfolding and its possible toxic effects [1–4]. Thus, protein instability caused by heat and/or osmotic shock induces the production of heat-shock proteins and the accumulation of osmolytes [5,6]. Similarly, the unfolded protein response in yeast is a consequence of the accumulation of unstable polypeptide intermediates in the endoplasmic reticulum [7].

Protein aggregation inside cells is far from being a simple two-state event, since the same protein may be deposited in cells as aggregates of different complexity [8–10]. Cells do not experience a simple soluble or

insoluble alternative, but are exposed to discrete classes of aggregates differing in the conformational state of the enclosed proteins [8–10]. Interestingly, evidences have been reported that differently structured aggregates have different effects on cells. For instance, in eukaryotes, the soluble aggregates of proteins responsible for neurodegenerative diseases are more toxic than larger assemblies [8].

Heterologous proteins expressed in *E. coli* often aggregate, because the folding capacity of the cell machinery may be limited by the non-physiological rates of expression. Cells react to this stress through complex and overlapping stress responses [1]. Among these, cells activate the expression of a set of proteins involved in removing the aggregates, such as chaperones, proteases and other folding catalysts [11]. After protein release from aggregates, refolding into their native state can take place [12–14].

In this work, we investigated whether the overexpression of recombinant proteins in bacteria induces responses other than the production/activation of folding helpers and whether the structure of protein aggregates has a role in eliciting these responses. In particular, we focussed on possible variations in the cell membrane, which was already reported to undergo rearrangements upon treatment with compounds able to mimic a heat-shock stress [15–17]. We, therefore, examined the effects of recombinant protein aggregates on cell membranes by choosing, as model systems, the glutathione-S-transferase (GST) and its fusion with the green fluorescent protein (GFP-GST), since it is possible to tune their aggregation by changing the expression conditions. In particular, it has been recently reported that GFP-GST can form soluble and insoluble aggregates of different

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; E₃, dihydrolipoyl dehydrogenase; EM, electron microscopy; FT-IR, Fourier transform infrared spectroscopy; GFP-GST, green fluorescent protein-glutathione-S-transferase; GST, glutathione-S-transferase; IB, inclusion body; IPTG, isopropyl β-D-1-thiogalactopyranoside; NPN, 1-N-phenyl-naphthylamine; pETM33-GST vector, GST under T7 promoter; PFL, *Pseudomonas fragi* lipase; pQE30-GST vector, GST under T5 promoter; SOD, superoxide dismutase; TLC, thin-layer chromatography

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complexity [9]. These experimental systems allow not only the study of the effects on the host cell membranes of a recombinant protein in soluble or aggregated form, but also those of the aggregate conformational states. Indeed, different aggregates have been already found to affect differently phospholipid bilayers [18]. To monitor the correlation among recombinant protein aggregates, cell physiology, and stress parameters, we employed, as a reporter probe, a beta-galactosidase gene expressed under the control of an *lbpB* promoter, specifically activated by misfolded proteins undergoing aggregation [11,19,20]. To study lipid changes occurring in intact cells during recombinant protein expression and aggregation, beside conventional biochemical methods, we employed Fourier-transform infrared (FT-IR) spectroscopy, a technique that has been widely used to characterize membrane properties and lipid composition [21–23].

Our results indicate that the folding state of the recombinant protein and the complexity of the intracellular aggregates critically affect the cell response, with regard to both the host proteome and the composition and permeability of the cell membrane.

2. Materials and methods

2.1. Cell culture, protein identification and aggregation analysis

Recombinant glutathione-S-transferase (GST) was expressed in *E. coli* from plasmids pQE30 (Qiagen; T5 promoter into M15 strain) and pETM33 (T7 promoter into BL21(DE3) strain) [24]. The green fluorescent protein–GST fusion protein (GFP–GST) was obtained using a Gateway vector (Invitrogen) [9]. Co-transformation with the *lbpB*-promoter/beta-galactosidase vector was performed to monitor in vivo the aggregation of the recombinant protein [11,19]. Bacteria were initially grown at 37 °C until the cultures reached an OD_{600 nm} of 0.4 and, subsequently, the growth temperature was adjusted in the different experiments to values ranging from 20 °C to 37 °C. Recombinant expression was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), when the OD_{600 nm} reached the value of 0.6. Culture samples were collected and fractionated as previously described [24]. The insoluble recombinant protein was quantified after SDS gel separation of the pellet recovered after centrifugation of the bacteria lysate from samples taken at different times during cell culture. The supernatant obtained after lysate centrifugation was used to load the 2D gels. Both 2D runs and mass spectrometry identification and quantification of the separated proteins were performed at the Proteomic Facility of EMBL-Heidelberg.

The sample preparation and the evaluation of the protein aggregation level by beta-galactosidase activity were described in detail in [19]. In particular, the activity of beta-galactosidase was determined spectrophotometrically and expressed in Miller units [25], according to: Miller units = $1000 \times (\text{OD}_{420} - 1.75 \times \text{OD}_{550}) / (t \times V \times \text{OD}_{600})$ where t = reaction time in minutes, V = volume of culture assayed in milliliters.

2.2. Electron microscopy

Protein samples (15 μM) were fixed by dropping 5 μL on a grid (Agar Scientific). After 1 min incubation at room temperature, the excess fluid was removed using filter paper and the unbound protein was washed before the grids were placed on a 50 μL drop of 1% uranyl acetate with the section side downwards. Finally, the grids were dried, placed in the grid-chamber and stored in desiccators before observation with a CM120 BioTwin electron microscope (Philips).

2.3. Cell membrane permeability and lipid composition

Cell membrane permeability was estimated analyzing the fluorescence variation of the hydrophobic probe 1-N-phenyl-1-naphthylamine (NPN) (Sigma), according to a protocol [15] modified from the original

method described by Helander and Mattila-Sandholm [26]. Recombinant protein expression was induced with 0.1 mM IPTG when the cell culture reached the OD₆₀₀ of 0.4. Bacteria were then grown at 37 °C for 90 min and then 200 μL of cell culture were collected and diluted 1:1 in 50 mM HEPES containing 5 μg/mL NPN. The uptake of the fluorescent probe was measured after 10 min incubation at 25 °C, setting the excitation and emission wavelength of the fluorimeter at 350 and 422 nm, respectively.

Membrane lipids were identified after extraction and separation by thin-layer chromatography (TLC) using lipid standards and silica coated TLC plates (Merck Eurolab). Bacteria cultured as described above were centrifuged and resuspended in a 20 fold lower volume of fresh LB medium to increase cell concentration. 1 mL of concentrated cell suspension was transferred into a screw-capped glass tube and 3.75 mL of a chloroform/methanol/concentrated HCl mixture (5:10:0.075) were added. After vortexing, 1 mL of chloroform was added and the mixture was vortexed again before supplying 1 mL of H₂O. The suspension was separated in three phases by centrifugation (2 min at 1500 ×g). The lowest phase containing the lipid fraction was recovered and after a second extraction in water the lipid fraction was transferred in a 1.5 mL silicon-coated tube. The solvents were evaporated in a vacuum centrifuge and the pellet resuspended in 10 μL of a 1:1 chloroform/methanol mixture. 1 mg of standard asolectin was resuspended using the same conditions. A thin layer chromatography plate was activated by incubation (30 min × 100 °C) in the presence of chloroform/methanol/glacial acetic acid/water (60:50:1:4) and the samples were loaded and separated. For their identification the plate was first dried, treated with molybdophosphoric acid and finally heated at 100 °C [27].

2.4. Cell membrane fluidity by fluorescence anisotropy measurements

Cell membrane fluidity was evaluated by measuring the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes) [28,29] on recombinant bacteria transformed with pQE30 or pETM33, before and 90 min after induction. Cells were washed twice with phosphate buffer saline (PBS, pH 7.4) and resuspended in the same buffer up to OD_{360 nm} = 0.07. DPH was added to the cell suspensions at a final concentration of 0.2 μM and its fluorescence was collected 40 min after incubation at 37 °C (determined insertion time). Steady state fluorescence was measured by a Perkin Elmer LS45 spectrofluorimeter with excitation and emission respectively at 360 nm and 450 nm (bandwidth 10 nm).

2.5. FT-IR microspectroscopy

Intact *E. coli* cells were recovered from the fermentation broth (1–2 mL), centrifuged (5000 rpm, 15 min, 4 °C), rinsed, and resuspended in water. 10 μL of this suspension were deposited on an infrared transparent support of BaF₂ and dried at room temperature for 30 min. The FT-IR absorption spectra were acquired in transmission using the UMA 500 infrared microscope coupled to a FTS40A spectrometer (Bio-Rad, Digilab Division, Cambridge, MA, USA) in the range 4000–800 cm⁻¹. A nitrogen cooled mercury cadmium-tellurium detector (narrow band, 250 μm) and the following conditions were employed: 2 cm⁻¹ spectral resolution, 20 kHz scan speed, 128–256 scan co-additions, triangular apodization. By setting the microscope square diaphragm aperture to 100 μm × 100 μm, a high-quality spectrum was collected within a few minutes.

In order to better identify the absorption bands of the lipid components, the second derivative analysis of the spectra was performed after a 15 point smoothing using the Savitzky-Golay method (3rd polynomial, 13 smoothing points) using the GRAMS/32 software (Galactic Industries Corporation, USA).

The FT-IR characterization of standard lipids purchased by Sigma was performed in attenuated total reflection on a single reflection

Fig. 1. Activation of the *lbpB*-promoter/beta-galactosidase reporter and variations of host cell permeability induced by recombinant protein aggregation. a) Aggregation of recombinant GST evaluated at different temperatures using the enzymatic reporter probe *lbpB*-promoter/beta-galactosidase. The beta-galactosidase activity was measured 90 min after recombinant induction in the host cells transformed with one of the two vectors (pQE30-GST –■–; pETM33-GST –□–) and the activity units calculated according to Miller [25]. Standard deviations from five experiments are not indicated when smaller than the symbol. b) Cell permeability measured in the above host strains before and 90 min after induction of GST expression at 37 °C. Data from three experiments.

diamond element (Golden Gate, Specac, UK) using the FTS40A spectrometer with a deuterated triglycine sulphate detector under the following conditions: 2 cm⁻¹ spectral resolution, 5 kHz scan speed, 256 scan co-additions, triangular apodization.

In the figures, we reported the measured spectra of one representative experiment. Standard deviation of peak position from three independent experiments was about 0.5 cm⁻¹, while standard deviation of band intensity was about 2%.

3. Results

3.1. Membrane lipid modifications induced by recombinant protein aggregation

To study the effects of recombinant protein aggregation on the cell membrane, we compared bacterial model systems expressing recombinant GST at different rates and monitored its aggregation using a specific probe, the *lbpB*-promoter/beta-galactosidase reporter [11,19]. The promoter of such a probe is specifically triggered by misfolded proteins undergoing aggregation [11,19]. In strains co-transformed with vectors carrying the probe and a sequence coding for a recombinant protein, the activity of beta-galactosidase indirectly enables to evaluate the level of recombinant protein misfolded and aggregated. In this system, we found that the recombinant GST protein was expressed mainly in the native form when bacteria, transformed with pETM33-GST vector, were kept for 90 min at temperatures between 20 °C and 37 °C, after induction of the T7 promoter (Fig. 1a). In contrast, GST was found to aggregate in bacteria kept at temperatures above 25 °C, when the T5-promoter pQE30-GST vector was employed (Fig. 1a) [19].

The permeability of the cell membrane was measured in the two expression systems 90 min after induction at 37 °C by means of a NPN fluorescent probe [15,26] (Fig. 1b). The permeability did not significantly change in the strain hosting soluble GST (from pETM33), while it slightly decreased of about 25% after induction in the strain characterized by high GST misfolding and aggregation (from pQE30).

The overall changes in membrane lipids were detected by TLC [27]. No significant variation was found in cells producing soluble GST from pETM33, whilst some alterations were observed in the strain expressing aggregated GST from pQE30, such as an increase of about 20% in the phosphatidyl glycerol content (data not shown). Although the lack of reliable standards for *E. coli* lipids didn't allow the identification of the specific molecules involved in this rearrangement, this analysis provided general hints that changes in the membrane lipids occur under aggregation stress.

In conclusion, it was found that the stress due to recombinant protein misfolding and aggregation induces a reduction of permeability and a change in the lipid content of the cell membranes.

3.2. Membrane lipid modifications detected by FT-IR spectroscopy

Modifications in the cell membranes were also analyzed by FT-IR spectroscopy. The absorption of infrared radiation by lipids enables to identify their chemical bonds, such as CH₂, CH₃, CH, and C=O and, therefore, to monitor their structural modifications [30]. The lipid absorption bands in the 1220–1468 cm⁻¹ region have been assigned in

Fig. 2. FT-IR spectra of intact bacterial cells expressing recombinant GST. a) Second derivative FT-IR spectrum of *E. coli* intact cells transformed with the pQE30-GST vector, 90 min after induction at 37 °C. b) Second derivative FT-IR spectra in the CH stretching region of host cells transformed with pETM33-GST (—) and pQE30-GST (·····) vectors, 90 min after recombinant induction at 37 °C. GST aggregation occurs at low and high levels, respectively, in the host cells transformed with pETM33-GST and pQE30-GST vectors. For comparison, derivative spectra were normalized at the 2956 cm⁻¹ CH₃ band. In the inset, the DPH fluorescence anisotropy for the above host cells, 90 min after induction at 37 °C, is reported.

in vitro studies [21,31]. However, the most useful infrared response for the study of lipids in intact cells is that of the CH, CH₂, and CH₃ bonds between 3050 cm⁻¹ and 2800 cm⁻¹, since no important absorption bands of other cell components overlap in this region. The infrared absorption spectra of standard lipids in the above mentioned spectra regions are reported in the [Supplementary Fig. 1](#).

In [Fig. 2](#), the second derivatives of the FT-IR absorption spectra of the *E. coli* host cells, transformed with pETM33-GST and pQE30-GST vectors, are reported. This mathematical procedure, widely used to resolve overlapping bands, allows identification of the different components in the absorption spectrum [32]. Furthermore, the relative weight of these components can be evaluated by the intensity of the negative peaks in the second derivative spectrum [32]. In [Fig. 2a](#), the second derivative spectrum of the host cells is presented in the lipid absorption region from 3050 to 2800 cm⁻¹, as well as in the protein Amide I and Amide II region from 1700 to 1500 cm⁻¹. These last bands reflect the protein secondary structures and their intermolecular interactions [32,33] and have been extensively examined for the study of protein aggregation in vivo [34–39]. In the present work we extended the FT-IR investigation to the lipid absorption due to acyl chains, in particular the two CH₂ peaks around 2923 cm⁻¹ and 2853 cm⁻¹, respectively assigned to asymmetric and symmetric stretching vibrations [21,30,40].

In [Fig. 2b](#) the second derivative spectra of host cells producing, at 37 °C, GST in soluble native form (pETM33) and in form of aggregates (pQE30) are reported. In presence of aggregation, we observed a higher intensity of the CH₂ bands in the spectrum of pQE30-GST transformed cells ([Fig. 2b](#)). This increased intensity reflects the presence of longer and/or more saturated acyl chains that can be responsible for a decreased membrane permeability ([Fig. 1b](#)) and fluidity [21,41]. This last hypothesis was supported by measurements of DPH fluorescence anisotropy of the two strains, 90 min after induction (inset of [Fig. 2b](#)). pQE30-GST transformed cells displayed a higher value of anisotropy than pETM33-GST cells, corresponding to a lower membrane fluidity [42].

The direct relationship between increased stress due to recombinant protein misfolding and aggregation and a higher intensity of the CH₂ lipid marker bands was confirmed by the comparison of the spectral response of pQE30-GST cells grown at 37 °C and at 20 °C ([Supplemental Fig. 2a](#)). The higher CH₂ band was observed at the highest expression temperature, when the *lbpB*-promoter/beta-galactosidase reporter is strongly activated, indicating higher aggregate accumulation ([Fig. 1a](#)).

It is interesting to compare these results with those obtained in other model systems such as *E. coli* expressing *Pseudomonas fragi* lipase (PFL) at 27 °C and 37 °C [36] ([Suppl. Fig. 2b](#)). In this case the CH₂ band intensity was lower at the highest temperature when the protein is expressed only in form of inclusion bodies (IBs), as it will be discussed later.

3.3. Host protein variations induced by recombinant protein aggregation

The effect of recombinant proteins expression on the host cell metabolism has been widely investigated [1]. However, no particular attention has been devoted to the relationship between the physical state of the recombinant proteins and the host cell metabolism. We, therefore, decided to explore whether such metabolic modifications correlate with the accumulation and the physical state of the recombinant protein aggregates. To this aim, we studied a strain expressing a GFP-GST construct that accumulates in aggregates, the complexity of which can be tuned modifying the growth temperature [9]. 1D and 2D gel electrophoresis of recombinant bacteria expressing GFP-GST in either soluble or aggregated form were examined and proteins identified by mass spectrometry.

The accumulation of recombinant aggregates was found to induce changes in the content of specific proteins involved in energy

Table 1

Variations of host cell protein content during the recombinant expression of GFP-GST

Host proteins	Content variations (%)			
	20 °C		37 °C	
	Hours after induction		Hours after induction	
	1 h	7 h	1 h	7 h
Dihydrolipoyl dehydrogenase (E ₃)	104±7	105±14	317±58	102±11
Glycerophosphoryl-diester phosphodiesterase	47±9	98±19	27±6	40±8
OmpA	331±38	186±41	526±77	389±66
IbpB	168±31	93±6	430±60	89±15
SOD	99±16	98±7	479±39	116±28

Protein content variations were evaluated from spots separated by 2D gel and identified by mass spectrometry of recombinant and wild type bacteria. OmpA variations were evaluated from the intensity of Coomassie stained bands separated in 1D SDS-PAGE and measured by densitometry. *E. coli* cells were examined 1 and 7 h after recombinant induction, grown at 20 °C and at 37 °C. Content variations are expressed as percentage of the values in non-induced cells (100%). Data are from three independent experiments.

metabolism, transcription, folding, and quality control (data not shown), as previously reported in literature for other recombinant systems [143].

In addition, in our work we report that the intracellular levels of some specific proteins – listed in [Table 1](#) – were found to change significantly after induction of the recombinant protein in the strain grown at 20 °C as well as at 37 °C. In particular, IbpB is a known marker of protein aggregation [13], OmpA is one of the major membrane proteins [44], dihydrolipoyl dehydrogenase (E₃) and glycerophosphoryl-diester phosphodiesterase are involved in the lipid and antioxidant metabolism [45]. The accumulation of superoxide dismutase (SOD) further suggests a transient oxidative stress ([Table 1](#)).

These results, therefore, indicate that recombinant protein aggregation affects the expression of host proteins involved in lipid metabolism and oxidative stress in the GFP-GST model system examined here.

3.4. Time course of GFP-GST aggregation and correlation with host protein and membrane modifications

In an attempt to correlate the variations in the host proteins with the GFP-GST misfolding and aggregation, we followed in parallel the evolution of the protein levels ([Table 1](#)) and of the complexity of aggregates detected by electron microscopy (EM) and by the activation of the *IbpB* promoter ([Fig. 3](#)).

The level of the host proteins reported in [Table 1](#) displayed a maximum variation in the first hour after GFP-GST induction and reached again their initial value 7 h later. In particular, the variation of the IbpB concentration estimated by densitometry on the 1D and 2D gels ([Table 1](#)) was confirmed by the *IbpB*-promoter-dependent beta-galactosidase activity ([Fig. 3](#)). In fact, the IbpB concentration increased in the first hour after the GFP-GST expression induction and then decreased during the following 6 h ([Table 1](#)).

The time dependence of the beta-galactosidase activity reported in [Fig. 3](#) indicated a rapid increase of protein misfolding [11] soon after GFP-GST induction, followed by a significant decrease in the next 6 h ([Fig. 3](#)). However, when the time course of beta-galactosidase activity is compared with IB formation ([Fig. 3](#)), an inverse correlation is observed, suggesting that misfolded proteins are entrapped in IBs. To better characterize this behaviour, the physical state of the protein 6 h after induction was investigated by EM ([Fig. 3](#)). Interestingly, not only insoluble structured nets were observed, but also small aggregates were found in the soluble fraction, showing the formation of soluble aggregates, as previously reported for the same system [9]. These results, therefore, indicated that the *IbpB*-promoter/beta-galactosidase reporter is activated in presence of the misfolded protein as well as of the soluble protein

Fig. 3. Time course of recombinant GFP-GST aggregation. The aggregate content, after GFP-GST induction at 20 °C, was estimated by the beta-galactosidase activity (B-gal), calculated as activity units according to Miller [25]. Insoluble aggregates were recovered after cell lysis and separated by SDS-PAGE. Their content was quantified by gel densitometry as relative amount in comparison to the densitometric highest value (IBs ▲). Protein aggregates present in the soluble fraction and in the pellet were identified by electron microscopy 6 h after induction. The control corresponds to an empty grid submitted to chemical fixation. The data refer to a single experiment; their standard errors from three independent experiments are for beta-galactosidase activity about 30 units and for IB about 7%. The OD₆₀₀ (◇) of the bacterial cell culture grown at 20 °C was measured for 24 h. The arrow indicates the time of induction.

aggregates. This behaviour can be explained suggesting a possible model of aggregation of the recombinant protein: soon after induction, misfolded GFP-GST forms first soluble aggregates – capable to activate the *lbpB* promoter –, which then precipitate into insoluble IBs. These, in turn, are slowly partially disrupted in the final phase of the cell culture. Indeed, we found a reduction of insoluble aggregates (IBs) between 7 and 20 h after induction, as shown in Fig. 3. In this last interval of time, we observed also a simultaneous increase of beta-galactosidase activity, suggesting that part of the protein released from IBs can be found in a misfolded form and in soluble aggregates.

The time dependence of the biochemical response presented above was found to be in agreement with the host cell lipid modifications monitored by FT-IR spectroscopy (Fig. 4). The intensity of the two CH₂ components slightly increased 1 h after induction and decreased to a minimum value after six more hours, namely at the moment of minimum soluble aggregate content and maximal insoluble protein accumulation (Figs. 3 and 4, inset). The infrared CH₂ bands increased again between 7 and 24 h, simultaneously with the decrease of insoluble aggregates and the increase of the beta-galactosidase activity (Figs. 3 and 4). We should note that the intensity of the two CH₂ peaks at 2923 cm⁻¹ and 2853 cm⁻¹, assigned respectively to the asymmetric and symmetric CH₂ stretching modes of lipid acyl chains, changed always in accordance at the same time, further supporting the significance of the spectral response.

The temporal evolution of host protein levels (Table 1), IB formation and *lbpB*-promoter-dependent beta-galactosidase activity (Fig. 3), indicate, therefore, that misfolded protein and soluble aggregates (but not the soluble native protein nor IBs) are responsible for the stress-dependent modification of the level of the host proteins and for the activation of the *lbpB*-promoter/beta-galactosidase reporter. Furthermore, the intensity variation of the CH₂ infrared bands was found to be correlated not with the presence of IBs, but

rather with that of the misfolded protein and soluble aggregates, probed by the *lbpB*-promoter/beta-galactosidase reporter.

4. Discussion

The present study on bacteria expressing recombinant proteins in native, misfolded, and aggregated form indicates that protein

Fig. 4. Lipid modifications during the expression of recombinant GFP-GST monitored by FT-IR spectroscopy. Second derivative absorption spectra of intact cells expressing GFP-GST, immediately and 1, 7, and 24 h after induction at 20 °C. Derivative spectra were normalized at the 2956 cm⁻¹ CH₃ band. The inset shows the SDS-PAGE of GFP-GST samples recovered at different times (H: hours) after induction: insoluble fraction (P) and total lysate (L).

misfolding and aggregation lead to a significant reorganization of the cell membrane, as well as of the host protein expression, sharing several features with other stress responses.

Interestingly, some of the host proteins whose level is affected by aggregation of the recombinant protein could be involved in lipid metabolism (Table 1). Indeed, small heat shock proteins like IbpB, known as protein aggregation markers [13], were found to stabilize cell membranes and to modulate their polymorphism [21]. It has been suggested that the role of small heat shock proteins could be crucial in the initial stage of the response to stress conditions that can alter the membrane structure [21]. The folding of OmpA is strongly dependent on the properties of the lipid bilayer [46] and it has been reported to have a role in membrane stabilization [44,47,48]. In addition, the temporary accumulation of E₃ could be an indication of a channelling of pyruvate into acetyl-CoA. Such a metabolite could be used not only for energetic needs, but also for lipid anabolism. In addition, E₃ is also involved in the antioxidant metabolism and, together with IbpB and SOD (Table 1), may participate in maintaining the membrane integrity preventing lipid peroxidation. Similarly to what happens in mammalian cells, the antioxidant activity of SOD could act together with the stabilizing and anti peroxidative activity of IbpB [49,50]. Summarizing, beside the classical activation of IbpA/B chaperones, the accumulation of recombinant protein aggregates seems: a) to trigger a transient oxidative stress as suggested by the increased levels of SOD and E₃; b) to stabilize the host membrane and regulate the porin-mediated transport, as indicated by the lower fluidity and by the accumulation of OmpA. It would be also interesting to investigate, in the presence of aggregation, the behaviour of proteins known for their role in membrane stress, such as RseB and phage shock protein A, which are respectively involved in sensing membrane stress [51] and in maintaining the membrane potential [52].

We also found that protein misfolding and aggregation induce rearrangements of the membrane and lipid composition in the host cells. In particular, we found a reduction of the membrane permeability (as shown by NPN fluorescence) and of fluidity (indicated by DPH fluorescence anisotropy). This behaviour is similar to that determined by heat-shock or benzyl alcohol treatments, which induce a lower permeability of the membrane after an initial hyperfluidification [15]. Furthermore, lipid modifications were detected by TLC and by FT-IR spectroscopy. In particular, the increase of the CH₂ infrared band intensities indicated the presence of longer and/or more saturated acyl chains under aggregation stress conditions.

The most striking understanding of the above observations can be derived from the comparison of the temporal evolution of the host protein levels (Table 1) and the lipid modifications (Fig. 4) with that of the aggregation process monitored by IB formation, EM, and IbpB-promoter-dependent beta-galactosidase activity (Fig. 3).

Indeed, all these results point out that misfolded protein and soluble aggregates (but not the soluble native protein nor IBs) activate the IbpB-promoter/beta-galactosidase reporter and are responsible for the membrane modifications and the changes in host protein levels.

Interestingly, native recombinant protein and large insoluble aggregates do not seem to appreciably activate stress markers (Table 1, Fig. 1a, Fig. 3) and membrane rearrangement (Fig. 1b, Fig. 2, Fig. 4, and Suppl. Fig. 2b).

In eukaryotic systems, pre-fibrillar aggregates, which have been found to be more toxic than large amyloids [8,53], are deposited into aggresomes to prevent their toxicity [54]. In this view, the possibility to store the overexpressed protein in insoluble aggregates could be a mechanism of the host cell to trap harmful species of misfolded proteins [8].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbapap.2008.10.015.

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