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Effects of recombinant protein misfolding and aggregation on bacterial membranes

D. Ami [a,b], A. Natalello [a,c], T. Schultz [d], P. Gatti-Lafranconi [a], M. Lotti [a], S.M. Doglia [a,c,*], A. de Marco [b,d,*]

[a] Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milan, Italy
[b] IFOM-IEO Campus for Oncogenomics, via Adamello 16, 20139 Milan, Italy
[c] Consorzio Nazionale Interuniversitario per le Scienze fisiche della Materia (CNISM) UdR Milano-Bicocca, via Cozzi 53, 20125 Milan, Italy
[d] Scientific Core Facilities, EMBL, Meyerhofstr. 1, D-69117 Heidelberg, Germany

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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.

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; E_3, dihidrolipoyl dehydrogenase E_3; 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-phenylnaphtylamine; 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

* Corresponding authors. S.M. Doglia is to be contacted at tel.: +39 02 6448 3459; fax: +39 02 6448 3565. A. de Marco, tel.: +39 02 574 303 229; fax: +39 02 574 303.
E-mail addresses: silviamaria.doglia@unimib.it (S.M. Doglia), ario.demarco@ifom-ieo-campus.it (A. de Marco).

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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 *IbpB* 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 *IbpB*-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: $\text{Miller units} = 1000 \times (OD_{420} - 1.75 \times OD_{550}) / (t \times V \times OD_{600})$ 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-phenylnaphtylamine (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 OD_{600} 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 H_2O. 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 molybdatphosphoric 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 BaF_2 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