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Protein aggregation and inclusion body formation in *Escherichia coli rpoH* mutant defective in heat shock protein induction

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Mutations in the *rpoH* gene, encoding σ^{32}, an alternative factor required for transcription of the heat shock genes, result in the extensive aggregation of virtually all cellular proteins and formation of inclusion bodies both under stress and non-stress conditions Inhibitors of protein synthesis suppress this aggregation, suggesting that newly synthesized proteins preferentially aggregate in *rpoH* mutants These data suggest that the heat shock proteins are involved in acquisition of the soluble state (i e correct conformation) of the bulk of intracellular proteins after their translation

Heat shock protein, Inclusion body, Protein folding, *rpoH* mutant, Chaperone

1. INTRODUCTION

The folding of proteins within living cells has been considered for many years as a spontaneous process [1] Current studies recognized auxiliary proteins called chaperones which are needed for protein folding, assembly and transport [2–4]. These proteins belong to the heat shock proteins (hsps) and constitute at least two protein folding systems· hsp70, represented in *E. coli* by DnaK, and hsp60, represented by GroEL [4] These systems have been already implicated in acquisition of active conformation of certain proteins [5–9], in promoting folding and assembly of proteins inserted into mitochondria [10–12], and in binding of newly synthesized polypeptide chains [13,14] However, it remains unclear how general their role is are they involved in the folding of the majority or only certain proteins? We address these questions using *E. coli* mutant in *rpoH* gene coding for RNA polymerase σ^{32} subunit responsible for the heat shock promoter recognition [15–18]. These cells were defective in the induction of hsps We find that virtually all proteins aggregate extensively and form inclusion bodies in *rpoH* mutant cells.

2. MATERIALS AND METHODS

2 1 *Strains and plasmids*

We used strains SC122, F-, *lac(am)*, *trp(am)*, *pho(am)*, *supC(ts)*, *strA*, *mal(am)*, and K165 SC122, *rpoH165* [19] Plasmid pRpoH is pDS2 [20]

2 2 *Media and growth conditions*

Strains were grown in L-broth at 30°C to the mid-logarithmic phase The cells were then incubated at 42°C or at 30°C for 1 h For plasmid-containing cells ampicillin (100 μg/ml) was added to the medium Viability of the cells after heat treatment was evaluated by plating at 30°C

2 3 *Preparation and fractionation of cell lysates*

Cells were chilled on ice, collected by centrifugation, and disrupted by sonication (4–5 10-s bursts) on ice in Laemmli loading buffer [21] without SDS, containing PMSF at the concentration 200 μg/ml The extent of cell disruption was monitored by phase contrast microscopy Usually less than 0 1% of cells remained intact after sonication The cell lysates were centrifuged in Microfuge 11 (Beckman) at 13 500 rpm for 2 min SDS was added to the supernatant The pellet was rinsed once with excess of the buffer, centrifuged and resuspended in SDS-containing loading buffer Before electrophoresis the samples were heated in a boiling water bath for 1–2 min Equal volume of soluble (S) and insoluble (I) protein fractions were separated on 10% SDS-PAGE [21] and Coomassie stained

2 4 *Microscopic techniques*

Cells were fixed for 30 min in 1% glutaraldehyde, 0 1 M Na-cacodylate, pH 7 3, at ambient temperature Fixation in 2% osmium tetroxide, 0 2 M Na-cacodylate, pH 7 3 was for 12 h at 4°C Fixed cells were treated with 0 5% uranyl acetate in maleate buffer, pH 5 2 for 90 min at 4°C The bacteria were then embedded in Epon Thin sections (less than 500 A) were stained with lead citrate [22,23]

3 RESULTS

E coli strain K165 [19] carries an amber mutation in the *rpoH* gene, which encodes the heat shock sigma factor [16–18] and a temperature-sensitive amber suppressor. At 30°C K165 grows normally despite the reduced amount of hsps [24, 25], but has limited ability to induce hsps synthesis. At 42°C the mutant cells are unable to form colonies, presumably due to cessation of hsps synthesis However, they continue to grow for at least 1 h (as judged by culture turbidity) and retain 100% viability during this time period, but fail to divide (data not shown) We incubated cells at 30°C or 42°C,

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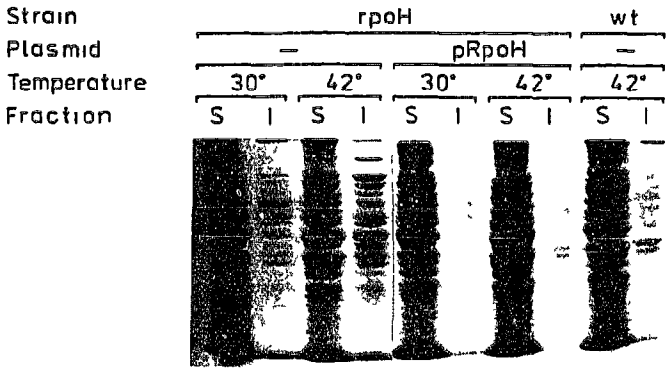

Fig 1 Protein aggregation in *rpoH165* mutant at 42°C Cells were grown at 30°C and portions of the cultures were transferred to 42° for 1 h Soluble (S) and insoluble (I) fractions were obtained as described in Materials and Methods and separated on 10% SDS-PAGE Note that insoluble fractions were loaded on gel in twice the amount of the corresponding soluble fraction *rpoH*= K165 strain, wt = SC122 strain

disrupted them by sonication, and fractionated the sonicate into soluble and insoluble fractions by brief centrifugation. Both fractions were analyzed by SDS- polyacrylamide electrophoresis (Fig. 1) We found that *rpoH165* cells had much more proteins in the insoluble fraction than did the wild-type cells. During incubation at 42°C the amount of protein in the pellet fraction of *rpoH* cells increased and reached 20–25% of total protein content after 1 h. Introduction of a plasmid carrying a wild-type *rpoH* gene prevented protein aggregation in the *rpoH* mutant strain (Fig. 1)

Phase contrast light microscopy revealed the appearance of refractive particles in *rpoH165* cells after heating (data not shown) Electron microscopic examination of thin sections of *rpoH165* heat-treated cells showed that aggregated proteins formed typical inclusion bodies (Fig 2), previously described for cells overproducing certain individual proteins [26] and for cells producing abnormal proteins in the presence of puromycin or amino acid analogues [27] Thus our data show that normal proteins behave like abnormal proteins under conditions of hsps shortage

No protein aggregation was observed when *rpoH165* cells were incubated at 42°C in the presence of antibiotic inhibitors of protein or RNA synthesis, while an antibiotic inhibiting DNA synthesis did not prevent aggregation (Fig. 3). These data indicate that aggregates are formed predominantly by newly synthesized proteins. Pulse labeling showed that protein synthesis did occur in *rpoH165* cells at 42°C at normal rates for at least 1 h. A substantial portion of the pulse-labeled proteins was insoluble (data not shown).

4 DISCUSSION

We observed extensive aggregation of proteins leading to inclusion body formation in *rpoH* mutant cells defective in hsps synthesis Previously inclusion body

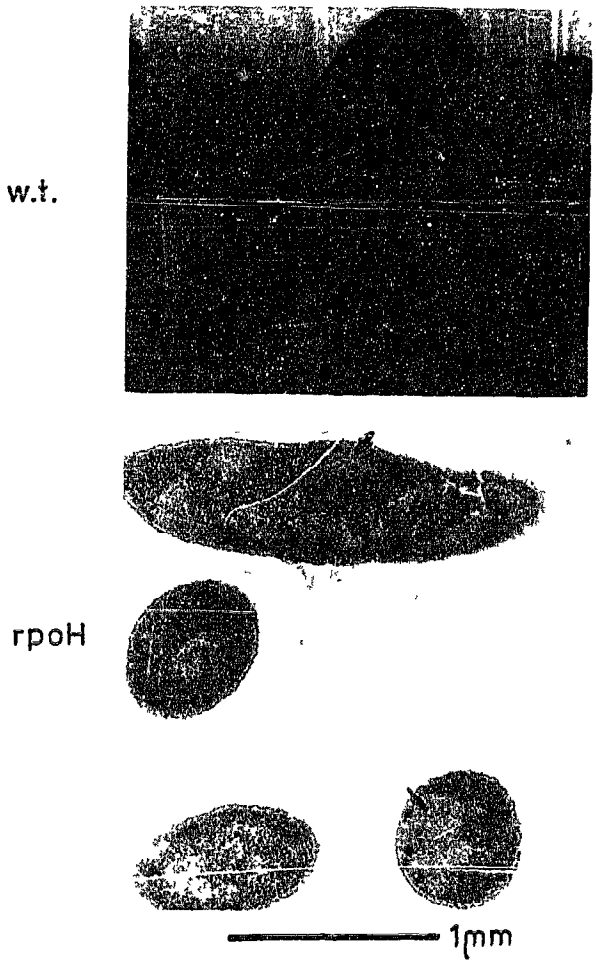

Fig 2 Electron micrographs of thin sections of *rpoH165* and wild-type cells Cells were incubated for 1 h at 42°C and thin sections were prepared as described in Materials and Methods Selected inclusion bodies are marked by arrows Wild-type = SC122 top, *rpoH165* = K165, bottom

formation was described only for overproduced [26] or abnormal proteins synthesized in the presence of puromycin or amino acid analogues [27] The most probable explanation of protein aggregation in the *rpoH* mutant is that some hsp(s) are necessary for correct folding and/or assembly of the bulk of newly synthesized polypeptides Conclusion that these are newly synthesized proteins that predominantly aggregate in *rpoH* mutant was based on the observation that inhibition of protein synthesis completely inhibited protein aggregation at the elevated temperature This suggests that most mature proteins are not damaged at 42°C in *rpoH* mutants. Since in certain well-studied cases folding intermediates were shown to be much more temperature-sensitive and prone to aggregation than mature proteins [28,29], one can speculate that these are folding intermediates of newly synthesized proteins which are forming aggregates in *rpoH* cells.

Protein aggregation in *rpoH* mutants was most pronounced at the elevated temperature of 42°C, but was

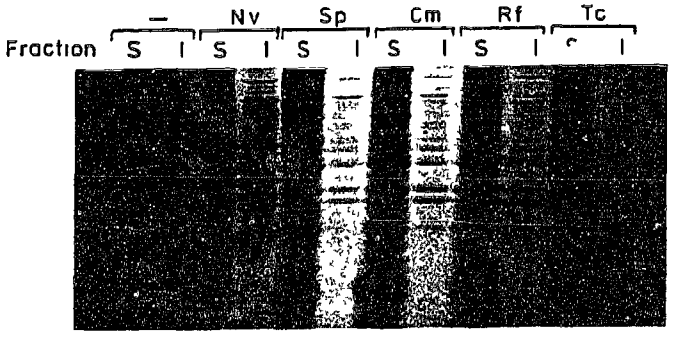

Fig 3 Effect of inhibitors on the protein aggregation at 42°C in the *rpoH165* mutant Cells were grown at 30°C and then shifted to 42°C as described in Materials and Methods with or without addition of antibiotics Nv = novobiocin (50 μg/ml), Sp = spectinomycin (50 μg/ml), Cm = chloramphenicol (20 μg/ml), Rf = rifampicin (50 μg/ml), Tc = tetracycline (20 μg/ml)

also readily detected at the non-stress temperature of 30°C It is known that some proteins retain solubility when overproduced in the wild-type cells, it appears, however, that these proteins (e g. β-galactosidase) became significantly aggregated in *rpoH* mutants even at 30°C (our unpublished data and [30]) This shows that the proposed general function of hsps is required not only under stress, but also under normal conditions, which is consistent with the indispensability of at least some hsps [31–34]

Which particular hsps protect proteins from aggregation in *E coli*? The obvious candidates are GroE and DnaR proteins (see Introduction) To test this we are performing experiments with *rpoH* mutants carrying plasmids with *groE* or *dnaK* operon

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