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Localization of DnaK and GroEL in *Vibrio cholerae*

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Abstract

Though the GroEL and DnaK heat shock proteins are well characterized in prokaryotes, only scanty and controversial information exist about their cellular localization. In the present study, the localization of the heat shock proteins DnaK and GroEL in normal and heat shocked cells of *Vibrio cholerae*, was investigated both by immunogold labeling of ultrathin sections and biochemical methods. Much of the DnaK was found to be localized at the inner membrane in unstressed cells, most probably at the Bayer's adhesion sites. Data suggested that upon heat shock, the DnaK associated with the membrane continued to remain there, but the newly synthesized DnaK appeared mostly in the cytoplasm. GroEL in both stressed and unstressed cells was found mainly in the cytoplasm.

Keywords: *Vibrio cholerae*; DnaK; GroEL; Localization

1. Introduction

All organisms, from bacteria to man, respond in a manner very similar to heat shock and other environmental stresses, by the rapid induction in synthesis of a set of heat shock proteins (Hsps) [1]. Some of these Hsps are highly conserved in evolution, especially those encoded by the *gro*EL (Hsp60) and *dna*K (Hsp70) genes [2–4]. The Hsp60 and Hsp70 proteins participate in refolding denatured polypeptides, preventing off-pathway aggregations [5] and facilitating protein translocation across membranes (reviewed in [6]). In eukaryotes, these functions are required within various cellular compartments, like mitochondria, endoplasmic reticulum, chloroplasts, nuclei, cytoplasm etc., accordingly, the Hsp60 and Hsp70 proteins are also found to be localized in these compartments [7]. Though there is a wealth of information on various aspects of Hsps and their regulation in prokaryotes, the information about their localization is limited. While the localization of DnaK has been studied comparatively extensively in *Escherichia coli*, there is very little information on other Hsps. A variety of experiments have indicated that DnaK in unstressed cells of *E. coli* is membrane associated [8–10]. On the other hand, the experiments of Bardwell et al. [11] demonstrated the presence of significant amounts of DnaK in the soluble fractions of wild-type cells. In an effort to resolve the controversy, Bukau et al. [12] used immunogold labeling of ultrathin sections of *E. coli* to examine the distribution of DnaK in both unstressed and heat shocked

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cells and found that in the former, most of the DnaK is cytoplasmic, though a fraction is localized near the membranes. No relocalization of DnaK upon heat shock was seen. In *Borrelia burgdorferi*, DnaK-like proteins have been found to be distributed both in the membrane and periplasmic fractions [13]. A similar observation has been made in case of a marine *Vibrio* species, namely *Vibrio* sp. strain S14 [14]. Information on the localization of GroEL exists for only one bacterial species *Helicobacter pylori* [15], where it has been shown that in the unstressed cells GroEL is localized primarily in the periplasmic region and on the cell surface. In an earlier communication, we presented our observations on the heat shock response in *V. cholerae* and showed by immunological and biochemical methods the induction of DnaK and GroEL in this organism subsequent to heat shock [16]. The present paper reports the results of our studies on the localization of these two proteins in normal as well as heat shocked *V. cholerae*.

2. Materials and methods

2.1. Biochemical procedures

Osmotic shock was done as described by Nossal and Heppel [17]. Logarithmically growing cells of *V. cholerae* El Tor MAK 757 [16] grown at 30°C, were harvested (5000×*g* for 4 min), washed once with 10 mM HEPES (pH 7.0) and were plasmolyzed at 16°C in 0.03 M Tris-HCl (pH 7.3)–20% (w/v) sucrose–0.2 mM EDTA and osmotically shocked in 0.3 mM $MgCl_2$ solution at 0°C. The shock fluid was separated from shocked cells by centrifugation at 10 000×*g* at 4°C for 10 min. The shocked cells were lyzed in 30 mM Tris-HCl (pH 6.8), by disruption using an ultrasonic disruptor (Sonicator Ultrasonic Processor XL, Heat Systems, NY, USA) (5 pulses of 15 s each at 7% efficiency) and centrifuged at 10 000×*g* for 10 min at 4°C. The supernatant constituted the shocked cell lysate.

Spheroplasts were prepared as described by Kaback [18] with minor modifications. Logarithmically growing cells of *V. cholerae* El Tor MAK 757 grown at 30°C were harvested by centrifugation at 5000×*g* for 4 min, the pellet was washed twice in 10 mM Tris-HCl (pH 8.0) at room temperature. Cells were suspended (1 g per 80 ml) at room temperature in 10 mM Tris-HCl (pH 8.0) and 10 mM EDTA (pH 8.0) containing 20% (w/v) sucrose. Lysozyme (Sigma, USA) solution in distilled water was added to it to a final concentration of 3 mg ml^{-1} and the suspension was incubated for 30 min at room temperature. Spheroplasts were pelleted down by centrifugation at 16 000×*g* for 10 min and suspended in 20% (w/v) sucrose and 10 mM EDTA by gentle pipetting. Supernatant was dialyzed against distilled water (three changes) at room temperature to remove sucrose. Spheroplasts were lyzed in 10 mM Tris-HCl (pH 6.8) by ultrasonication as described above. Unlyzed cells were removed by centrifugation at 16 000×*g* for 10 min at 4°C. NADH oxidase assay was performed as described by Orndorf and Dworkin [19]. β-Galactosidase assay was essentially according to Miller [20]. All protein estimations were done according to the method of Bradford [21]. For running protein gels, samples from above were dissolved in gel loading buffer, which contained 62.5 mM Tris-HCl (pH 6.8), 2.0% SDS, 25% glycerol and 0.01% Bromophenol blue. The samples were assayed on vertical SDS-polyacrylamide gel with 4% stacking and 10% resolving gel as previously described [16]. Silver staining was performed according to Damerval et al. [22]. Quantification of the intensity of protein bands was done by the densitometric analysis of dried gels with a laser densitometer (LKB Bromma 2202). The area under the peaks was determined and the relative amount of the protein in a particular fraction was calculated as percent of total protein. Heat shocking at 42°C of *V. cholerae* El Tor MAK 757 growing at 30°C was done as described previously [16]. All experiments were repeated at least three times.

2.2. Immunological methods

Immunogold labeling was performed essentially according to Culvenor et al. [23] with modifications as described. Bacteria were harvested by centrifugation (5000×*g* at 4°C), washed in ice-cold PBS (pH 7.4) [24] three times and were then suspended in the same buffer containing 0.5% each of paraformaldehyde and glutaraldehyde. Cells were immobilized by adding an equal volume of molten 2% low-melting-point agarose (Serva, Heidelberg, Germany) and im-

mediately spinning them in a microfuge. Suitable pieces were excised and dehydrated with a graded series of ethanol. These were then embedded in LR-white resin by polymerization at 55°C under vacuum for 24 h with a few changes in LR-white. Ultrathin sections cut with a Reichert ultracut S ultramicrotome (Leica Reichert Jung, Austria) were mounted on 200 mesh nickel grids. Non-specific binding sites were blocked with 1% BSA–0.01% Tween–20 in PBS (BSA-phos-Tween). Following washing in 0.1% BSA in PBS (washing buffer) the grids were incubated at 37°C for 2 h with anti-DnaK and anti-GroEL antibodies (obtained from Dr. Roger McMacken, Johns Hopkins University and Dr. Kathee Martincic, University of Pittsburgh, through the courtesy of Dr. D.K. Chattoraj, NIH, USA) at 1:500 and 1:300 dilution in BSA-phos-Tween, respectively. After a few washes in washing buffer, the grids were treated with goat anti-rabbit antibodies linked to Protein A gold 10 nm (Sigma, diluted 1:50 in BSA-phos-Tween) for 1 h at room temperature. The washings were done with washing buffer followed by double distilled water. The sections were then stained in 2% aqueous uranyl acetate for 1 h in the dark. Final washings were done in double distilled water. Negative control grids in which the incubation step with first specific antibody was omitted were processed in parallel to obtain the count of gold particles bound non-specifically to grids. The grids thus prepared were examined in JEOL 1200 EXII transmission electron microscope (TEM) (operating voltage, 60–80 kV).

3. Results and discussion

3.1. Release of DnaK by osmotic shock

The bacteria were osmotically shocked as described and the presence of DnaK and GroEL in the shock fluid and shocked cell lysate was examined by SDS-PAGE. For comparison, release of β-galactosidase a cytoplasmic enzyme in *V. cholerae* [25] and NADH oxidase, an enzyme tightly bound to inner membrane [19] were also examined. DnaK and GroEL proteins were identified as described before [16]. Assay of β-galactosidase and NADH oxidase was carried out as described in Section 2. The osmotic shock procedure, as described in Section 2, quantitatively releases most of the periplasmic proteins, while most of the cytoplasmic proteins or those bound tightly to the inner membrane remain within the cells. Under this condition, approximately 64% of soluble DnaK was found to be released by os-

Table 1
Gold particle count in *V. cholerae* cells immunolabeled with DnaK and GroEL specific antibodies

S. no.	Parameter	DnaK		GroEL	
		30°C	42°C	30°C	42°C
1	No. of cells examined[a]	34	59	27	42
2	Total no. of gold particles in cells[b]	1398	3278	646	1472
3	Gold particles in cells/gold particles in background[c]	17.11	41.91	12.94	28.39
4	Gold particles per cell[d]	41.11	55.55	23.92	35.04
		(28.47)	(39.60)	(17.46)	(24.29)
5	Gold particles at membrane per cell[e]	24.20	22.91	6.14	10.38
		(19.21)	(13.79)	(5.57)	(6.85)
6	Gold particles at cytoplasm per cell	16.91	32.64	17.77	24.66
		(15.25)	(26.83)	(15.00)	(19.52)
7	Gold particles at membrane (% of total)	58.86	41.24	25.66	29.62

[a]All cells that were in focus on the photographs were analyzed.
[b]Numbers represent the total of gold particles present in the cells analyzed. All gold particles were considered which were located within the cells or outside the cells up to a distance of two gold particles from the membranes.
[c]Determined by calculating the number of gold particles bound to an equivalent area of cells in samples and negative controls, to obtain the number of gold particles specifically bound to DnaK/GroEL antibodies.
[d]The standard deviation is given in parentheses.
[e]Gold particles located at the membranes or up to two gold particles away from either side of the membranes were considered as membrane associated.

motic shock, compared to 34% of GroEL (Fig. 1A), indicating that a substantial portion of the DnaK protein is localized in the periplasm. Corresponding values for β-galactosidase and NADH oxidase were 21 and 8%, respectively (Fig. 2).

3.2. Retention of DnaK inside spheroplasts

To examine further whether the DnaK and GroEL are present in periplasm or in cytosol, release of these proteins from spheroplasts was examined. Spheroplasts were prepared as described in Section 2 and the presence of DnaK in the supernatant fraction and within the spheroplasts was examined by SDS-PAGE. Preparation of spheroplasts by lysozyme-EDTA treatment disrupts the outer envelope of the cell and exposes the periplasmic space to the external environment. Therefore, under this condition, any protein which is normally present in the periplasmic space should be found in the supernatant

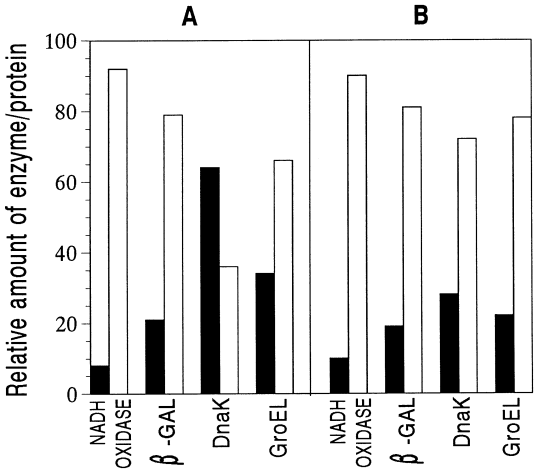

Fig. 2. The relative percentages of the NADH oxidase, β-galactosidase (β-GAL), DnaK and GroEL, calculated from three independent experiments are shown. (A) Osmotic shock experiment: shock fluid (solid bars) and shocked cell lysate (open bars). (B) Sheroplast experiment: spheroplast supernatant (solid bars) and spheroplast lysate (open bars).

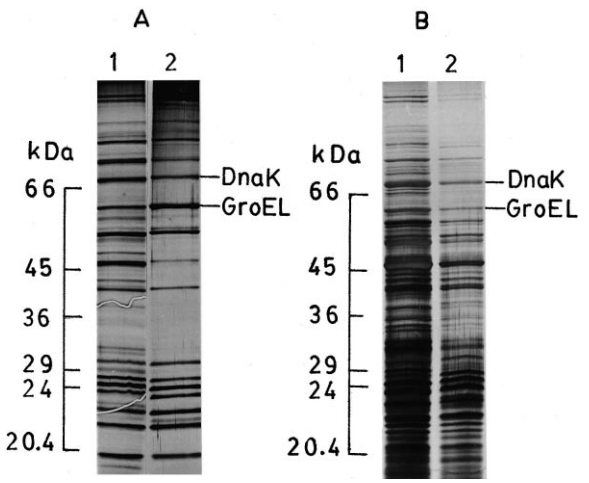

Fig. 1. (A) Osmotic-shock release of DnaK. Lane 1, osmotic-shock supernatant; lane 2, the shocked cell lysate. (B) Retention of DnaK and GroEL in spheroplasts. Lane 1, spheroplast lysate obtained by sonication; lane 2, spheroplast supernatant. The presence of some DnaK and GroEL in lane 2 was due to slight lysis of the spheroplasts during handling, which could not be controlled in repeated experiments. Position of molecular weight markers (Sigma) run concurrently on the gel are shown on the left: bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3 phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soyabean trypsin inhibitor (doublet, 20.4 and 19.7 kDa). Six micrograms of protein was loaded in each lane.

fraction. Examination of the gel pattern (Fig. 1B) revealed that most of the DnaK (72%) was retained within the spheroplast in apparent contradiction with the result obtained by the osmotic shock experiment. In contrast, almost identical results, with both sets of experiments (Osmotic shock and spheroplast assay), were obtained with GroEL, β-galactosidase and NADH oxidase indicating that GroEL is either localized in the cytoplasm or in the inner membrane. However, compared to GroEL, somewhat lesser amounts of β-galactosidase or NADH oxidase appeared to be released in osmotic shock and in spheroplast assays. Since very different methods for estimating the release of GroEL on one hand and β-galactosidase and NADH oxidase on the other were used, it was not possible to ascertain if this difference was genuine or simply a reflection of the different sensitivities of the methods used (Fig. 1BFig. 2).

It has been suggested that proteins which are associated with the inner membrane of cells in those regions where inner and external membranes are contiguous (Bayer's adhesion sites [26,27]) are released by extrusion, when cells are subjected to osmotic shock. It thus seems very likely that DnaK in *V. cholerae* is a protein which is localized primarily

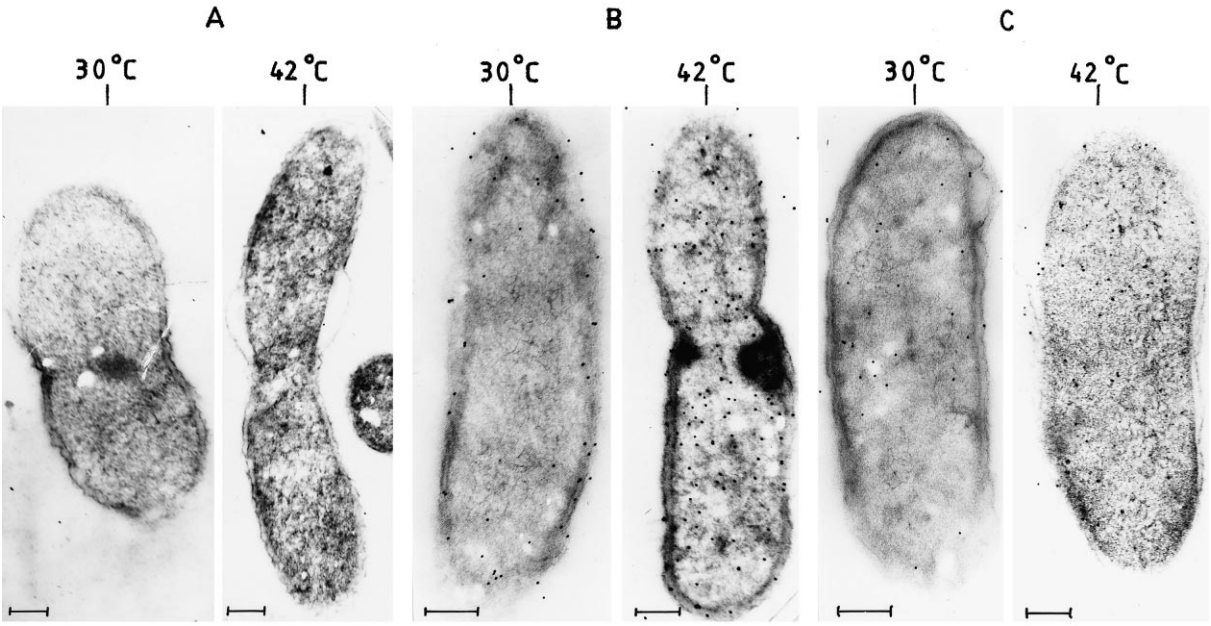

Fig. 3. Immunogold staining of *V. cholerae* MAK 757 before and after heat shock: (A) negative control (no incubation with specific antibody; see text for further details; (B) with DnaK specific antibodies; (C) with GroEL specific antibodies. Scale bar: 0.2 μm.

at Bayer's adhesion sites, i.e. at regions where the inner and outer membrane are contiguous. It can be mentioned here that recent experiments of Yaagoubi et al. [27] indicate that even in *E. coli*, about 20% of the DnaK molecules are located at such regions.

3.3. Immunogold analysis

The problem of localization of DnaK and GroEL proteins was further studied by immunogold electron microscopy. *V. cholerae* cells were grown at 30°C and immunogold localization of DnaK and GroEL was done by electron microscopy as described in Section 2. Fifty-nine percent (59%) of the DnaK bound gold particles were found to be localized predominantly on the inner membrane (Table 1 and Fig. 3B), in broad agreement with the results obtained by biochemical methods. All gold particles were considered which were located within or outside the cells up to a distance of two gold particles from the membranes [12]. Arguments have been presented, on theoretical grounds, that in the immunogold labeling experiments, about 20–30% molecules of a given cytoplasmic protein would be labeled with gold particles in a manner, which would make them indistinguishable from a membrane associated protein [12]. In the case of *V. cholerae*, even after making allowance for this, about 30–40% of the DnaK molecules were found to be membrane associated. When the cells were shocked to 42°C, there was an increase in the total number of DnaK proteins but it appeared that most of the newly synthesized DnaK was localized in the cytoplasm (Table 1 and Fig. 3B). This observation was unlike that in *E. coli* where immunogold localization of DnaK showed that it is present predominantly in the cytoplasm in both stressed and unstressed cells [12]. It ought to be mentioned here that this method cannot distinguish between the proteins associated with the membrane and those which are present at the Bayer's adhesion junctions. The presence of DnaK at Bayer's adhesion sites on the inner membrane is perhaps not very surprising, as this protein has been implicated in protein export [28], besides in a number of other functions in this organism, all of which require membrane association. Further, DnaK is known to function in conjunction with DnaJ [29],

which is reported to reside exclusively in the cell envelope [30]. Though nothing is known about the role of DnaK in *V. cholerae*, it is very likely that this protein plays similar roles both in *V. cholerae* and in *E. coli*. It is well known that *V. cholerae* has a very efficient protein export machinery which is responsible for the secretion of a large number of proteins important for the organism's pathogenicity and survival in diverse environments [31,32]. It is possible that DnaK plays a more significant role in protein export in *V. cholerae* than it does in *E. coli* and the higher level of membrane association of DnaK in *V. cholerae* is a reflection of that. It is interesting to note in this connection that in another *Vibrio* species, namely *Vibrio* sp. strain S14, it has been shown recently that DnaK is found in the periplasm when these cells are subjected to a carbon-starvation stress [14].

Contrary to what was found for DnaK, much (about 74%) of the GroEL protein in 30°C grown *V. cholerae*, was present in the cytoplasm (Table 1) with no obvious clustering in any part of the cytoplasm (Fig. 3C), a result which agrees well with the biochemical data. This is in contrast to what is found in *H. pylori* where the GroEL protein is seen to be localized primarily on the cell surface and in the periplasmic region [15]. No evidence of any significant relocalization of GroEL could be seen upon heat shock (Table 1 and Fig. 3C). GroEL is required for protein folding [5] and in *E. coli* it has been shown to be associated with newly synthesized polypeptides released from the ribosomes [33], which are present in the cytoplasm. Our finding that GroEL is present predominantly in the cytoplasm, is thus in congruence with the above observation and indicates that it plays identical roles both in *V. cholerae* and *E. coli*.

Our results, taken together with what is known for other prokaryotes examined to date, thus seem to indicate that even though DnaK and GroEL play similar roles in these organisms, their distribution within the cells varies considerably.

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