Immunogold localization of the DnaK heat shock protein in *Escherichia coli* cells

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Previously reported cell fractionation experiments have yielded conflicting information on the cellular localization of the DnaK heat shock protein of *Escherichia coli*. Here we used immunogold labelling of ultra-thin sections to determine the localization of DnaK in unstressed cells at 30 °C as well as in heat-shocked cells. In cells grown at 30 °C, gold particles were found predominantly in the cytoplasm, indicating that the majority of the DnaK molecules are cytoplasmic; however, a fraction of the gold particles was located in proximity to the membranes, raising the possibility that a subpopulation of DnaK proteins is membrane-associated. Heat shock of the cells did not induce detectable relocation of DnaK.

Introduction

The DnaK heat shock protein of *Escherichia coli* is a member of the ubiquitous Hsp70 protein family that has been highly conserved during evolution (Bardwell & Craig, 1984). Hsp70 proteins are molecular chaperones that catalyse protein folding as well as disassembly of oligomeric protein complexes (Morimoto *et al.*, 1990; Gething & Sambrook, 1992). In eukaryotes, this chaperone function appears to be required within multiple cellular compartments since Hsp70 proteins are found in the mitochondria, chloroplasts, endoplasmic reticuli, nuclei and cytoplasm of several types of eukaryotic cells (Morimoto *et al.*, 1990; Gething & Sambrook, 1992). Furthermore, in *Drosophila*, cytoplasmic Hsp70 proteins relocate upon heat shock and migrate to the nucleus (Morimoto *et al.*, 1990), suggesting stress-induced changes in their cellular functions. In *E. coli*, conflicting data exist on the localization of DnaK in unstressed cells, while no information is available on its localization in heat-shocked cells. In minicells infected with lambda phages encoding the *dnaK*⁺ gene, DnaK was predominantly found in the membrane fraction (Zylicz *et al.*, 1983). Consistent with this result it has been shown that DnaK cofractionates with the inner membrane fraction of minicells (Kostyal *et al.*, 1989). In another study, it was reported that DnaK was not only associated

with membrane fractions but was also present in significant amounts in the soluble fractions of wild-type cells (Bardwell *et al.*, 1986). It is unclear whether the apparent association of DnaK with membrane fractions reflects a physiologically significant state or is an artifact of the cell lysis procedure. In fact, cell fractionation experiments have been shown to yield artifactual results with respect to the localization of hybrid proteins (Tomassen *et al.*, 1985). DnaK might be particularly prone to artifactual association with membranes during cell fractionation since, as a chaperone, it is capable of hydrophobic protein–protein interactions (Liberek *et al.*, 1988). Furthermore, non-esterified fatty acids are bound to Hsp70 of rat cells (Guidon & Hightower, 1986), which raises the possibility that Hsp70 proteins in general exhibit affinities to membrane fractions. In these experiments we used immunogold labelling of thin sections of *E. coli* cells to examine the cellular localization of DnaK in unstressed cells and to establish whether heat-shock-induced relocation occurs.

Methods

Generation of DnaK-specific antiserum. We purified DnaK according to standard procedures (Schlossman *et al.*, 1984) from cells over-producing DnaK due to the presence of the multicopy plasmid pBB1, which encodes the *dnaKJ* operon (unpublished data). DnaK was further gel-purified and injected into rabbits. The polyclonal antiserum was specific for DnaK but cross-reacted with other cellular proteins in *dnaK*⁺ and Δ *dnaK52* cells (Fig. 1, lanes 1 and 2). In order to eliminate cross-reactivity we first absorbed the antiserum to a Sepharose column

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Fig. 1. Specificity of antisera raised against DnaK. The specificity of a polyclonal rabbit antiserum raised against purified DnaK (lanes 1 and 2), and of DnaK-specific antibodies obtained by affinity purification of this serum (lanes 3 and 4), was tested by immunoblots using total cell extracts of wild-type cells (lanes 1 and 3) and of $\Delta dnaK52$ cells (lanes 2 and 4). The position of DnaK is indicated.

which had total cellular extracts of $\Delta dnaK52$ mutants covalently linked to it, then purified DnaK-specific antibodies by affinity chromatography with DnaK covalently linked to Sepharose. The resulting antiserum was very specific for DnaK and eliminated most cross-reactivity with other cellular proteins in $dnaK^+$ and $\Delta dnaK52$ cells (Fig. 1, lanes 3 and 4).

Preparation of cells for immunogold staining. Cells of *E. coli* strains AB1157 ($F^- thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 supE37$) and GW4813 (AB1157 $\Delta dnaK52::Cm^r$) (Paek & Walker, 1987) were grown at 30 °C in M9 minimal medium (Miller, 1972) supplemented with appropriate amino acids (50 $\mu g\ ml^{-1}$), thiamin (0.001%), and glucose (0.2%). Aliquots (2.5 ml) of exponentially growing cells ($OD_{600}=0.5$) were either incubated for 15 min at 43.5 °C or left at 30 °C, and then subjected to the fixation procedure.

Immunogold staining. Cells of strains AB1157 and GW4813 grown at 30 °C were fixed according to Francesconi *et al.* (1988) with modifications as follows. Cells were centrifuged for 2 min, immediately resuspended in 1.5 ml fixation mixture (2%, v/v, paraformaldehyde, 0.1% glutaraldehyde, 100 mM-cacodylate buffer pH 7.2), and incubated at room temperature (30 °C probes) or at 43.5 °C (heat-shocked probes) for 1 h, then at 4 °C for 5 h. The supernatant was withdrawn, replaced with 0.5 M-NH₄Cl (0.3 ml) and further incubated overnight. The cell pellet was dehydrated (Francesconi *et al.*, 1988) and embedded in Lowicryl K4M followed by UV polymerization (Simon *et al.*, 1987) according to standard procedures. Blocks (1 mm²) of 90 nm thickness were prepared and placed on 200-mesh nickel grids. For immunostaining, the grids were incubated for 20 min at room temperature in blocking buffer (phosphate-buffered saline, sodium azide, bovine serum albumin; EY Labs, Inc.), followed by overnight incubation at 4 °C with a 1:10 dilution of an anti-DnaK polyclonal antibody solution. After incubation with primary antibodies, the grids were rinsed six times for 10 min in blocking buffer and incubated for 60 min at room temperature with a 1:2 dilution of secondary antibodies (goat-anti-rabbit antibodies with covalently linked gold particles of 10 nm diameter; EY Labs, Inc.). The grids were rinsed three times for 10 min in blocking buffer and twice for 10 min in H₂O, followed by incubation in osmium tetroxide (2%, w/v) for 2 min. After rinsing three times for 5 min in H₂O the specimens were post-stained in a saturated solution of uranyl acetate (5 min) and Reynold's lead citrate (2 min). The samples were analysed at 80 kv in a JEOL 100B electron microscope.

Results and Discussion

An important requirement for our approach of using immunogold labelling to analyse the cellular localization of DnaK in *E. coli* cells was the availability of *dnaK* null mutants to serve as negative controls. We have recently reported the isolation and characterization of $\Delta dnaK52::Cm^r$ mutants which completely lack DnaK (Bukau & Walker, 1989*a,b*; 1990; Paek & Walker, 1987). Wild-type (AB1157) and $\Delta dnaK52::Cm^r$ mutant (GW4813) cells were grown at 30 °C and either subjected to a heat shock at 43.5 °C or left at 30 °C. Cells were fixed and incubated with a purified anti-DnaK polyclonal antibody solution that was highly specific for DnaK

Fig. 2. Immunogold staining of wild-type and $\Delta dnaK52$ mutant cells at 30 °C. Ultrathin sections of cells of strains AB1157 ($dnaK^+$) (a) and GW4813 ($\Delta dnaK52$) (b) grown at 30 °C were immunogold labelled using DnaK-specific antibodies and analysed by electron microscopy, as described in Methods. Bar, 0.5 μm .

Fig. 3. Immunogold staining of wild-type and $\Delta dnaK52$ mutant cells after heat shock. Cells of strains AB1157 (*dnaK*⁺) (a) and GW4813 ($\Delta dnaK52$) (b) grown at 30 °C were shifted to 43.5 °C for 15 min and ultrathin sections were prepared for immunogold labelling as described in Methods. Bar, 0.5 μ m.

Table 1. Gold particle counts of *E. coli* cells immunolabelled with DnaK-specific antibodies

	30 °C		42 °C	
	Wild-type	$\Delta dnaK52$	Wild-type	$\Delta dnaK52$
No. of cells*	31	66	72	45
Gold particles in cells†	222	182	1110	191
Gold particles in cells/gold particles in background‡	5.3	1.4	7.1	1.2
Gold particles per cell§	7.16 (6.63)	2.76 (3.96)	15.4 (14.84)	4.24 (5.99)
Gold particles in cytoplasm per cell	5.42 (5.75)	1.89 (3.31)	12.46 (13.08)	2.69 (5.36)
Gold particles at membranes per cell¶	1.74 (2.38)	0.86 (1.34)	2.95 (4.14)	1.56 (2.42)
Gold particles at membranes (% of total)	24.3	31.3	19.2	36.2

* All cells that were in focus on the photographs were analysed unless the cell envelope was disrupted or not detectable.

† Numbers represent the total of gold particles present in the cells analysed. 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. This distance corresponds approximately to the maximal theoretical distance (20 nm $\times$ magnification) that a gold particle can be away from its primary antibody bound to a membrane-associated antigen.

‡ For each analysed cell, gold particles present in a cell-free area of identical size and shape were counted as described in the preceding footnote. Cell-free areas were chosen that were adjacent to the corresponding cell at its right side or, if no cell-free area was present at this location, at the next cell-free location adjacent to the cell counterclockwise from its right side.

§ The standard deviation (σ) is given in parentheses.

|| Gold particles located within the cytoplasm of the cells up to a distance of two gold particles from the inside of the membranes were considered as cytoplasmic. The standard deviation (σ) is given in parentheses.

¶ Gold particles located at the membranes or up to two gold particles away from either side of the membranes were considered as membrane-associated. The standard deviation (σ) is given in parentheses.

(Fig. 1). After incubation with primary antibody, the cells were incubated with gold-labelled secondary antibodies and analysed microscopically.

The results of our localization study are shown in Figs. 2 and 3, and Table 1. The number of gold particles present within wild-type cells was well above (5.3-fold for unstressed cells; 7.1-fold for heat-shocked cells) the number of gold particles present within a comparable area of background. In contrast, the number of gold

particles present within $\Delta dnaK52$ control cells was only slightly (1.4-fold and 1.2-fold) above background. The slight increase in the number of gold particles within $\Delta dnaK52$ cells as compared to background might be due to cross-reactivity of the antibodies with cellular proteins other than DnaK which was not eliminated by purification of the anti-DnaK serum used here (see below). The results show that the immunogold labelling of wild-type cells is highly specific for DnaK.

In wild-type cells grown at 30 °C, the majority of the gold particles were located within the cytoplasm, indicating that DnaK is mostly cytoplasmic. We searched for clustering of gold particles within specific regions of the cytoplasm and especially within nucleoid structures, given that *ΔdnaK52* mutants possess severe defects in chromosome segregation (Bukau & Walker, 1989b), suggesting a direct or indirect role of DnaK in processes that are required for proper chromosome distribution. However, we did not observe significant clustering of gold particles within specific regions of the cytoplasm.

A significant subpopulation of gold particles was, however, located at or near the cell membranes, raising the possibility that a fraction of DnaK might be membrane-associated. Based on our studies we estimate that the maximum amount of DnaK that could possibly be membrane-associated is 24% of total DnaK (Table 1). This observed concentration of gold particles near the membrane, however, is also that predicted if there were no significant association of DnaK with the membrane. The estimate of maximum percentage of DnaK associated with the membrane was arrived at by calculating the percentage of cell-associated gold particles that were within two gold particles of the membranes. This distance corresponds approximately to the maximum theoretical distance that a gold particle could be from the membrane if its primary antibody was bound to a membrane-associated antigen and results in an area that is 20–30% of the total cellular area (depending on the orientation of the section). Therefore, for a given cytoplasmic protein, approximately 20–30% would be predicted to label with gold particles in a manner indistinguishable from that of a membrane-associated protein. Since this percentage is in the range of what was observed for DnaK, the percentage of DnaK molecules that is membrane-associated is probably significantly less than the maximum of 24% and allows the possibility that there is no significant localization of DnaK to the membrane at all. Finally, cross-reactivity to membrane-associated proteins other than DnaK might also contribute to a potential overestimation of the association of DnaK with the membrane. It has been reported that a region of the *E. coli* MOB2 membrane protein has significant homology to DnaK (Jacq *et al.*, 1989). Cross-reactivity to MOB2 might have remained undetected in the Western blot shown in Fig. 1 because of the small molecular mass (10 kDa) of MOB2. Taken together, our data do not support conclusions derived from cell-fractionation experiments that DnaK is largely membrane-associated in intact cells of *E. coli*.

In wild-type cells subjected to heat shock the number of gold particles per cell was more than twofold greater than in unstressed wild-type cells. The distribution of gold particles was similar to that in unstressed cells.

Thus, after heat shock the majority of DnaK molecules remain cytoplasmic and only a subfraction (up to 19%) of DnaK proteins might be membrane-associated. We found no indication of major stress-induced re-localization of DnaK.

In summary, our results indicate a predominantly cytoplasmic localization of DnaK in both unstressed and heat-shocked cells which suggests that the majority of the cellular processes that require chaperone functions of DnaK are cytoplasmic.

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