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NOTES

Localization of DnaK (Chaperone 70) from *Escherichia coli* in an Osmotic-Shock-Sensitive Compartment of the Cytoplasm

ABDELHAMID EL YAAGOUBI, MASAMICHI KOHIYAMA, AND GILBERT RICHARME*
Biochimie Génètique, Institut Jacques Monod, Université Paris 7, 75251 Paris Cedex 05, France

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The chaperone DnaK can be released (up to 40%) by osmotic shock, a procedure which is known to release the periplasmic proteins and a select group of cytoplasmic proteins (including thioredoxin and elongation factor Tu) possibly associated with the inner face of the inner membrane. As distinct from periplasmic proteins, DnaK is retained within spheroplasts prepared with lysozyme and EDTA. The ability to isolate DnaK with a membrane fraction prepared under gentle lysis conditions supports a peripheral association between DnaK and the cytoplasmic membrane. Furthermore, heat shock transiently increases the localization of DnaK in the osmotic-shock-sensitive compartment of the cytoplasm. We conclude that DnaK belongs to the select group of cytoplasmic proteins released by osmotic shock, which are possibly located at Bayer adhesion sites, where the inner and outer membranes are contiguous.

The heat shock protein DnaK/hsp70 (1, 8) plays a role in the maintenance of bacterial viability under stress, in addition to roles in normal cellular functions. It behaves as an ATP-dependent chaperone, facilitating the correct assembly or disassembly of some oligomeric protein complexes and participating in protein renaturation and folding and in the transmembrane targeting of certain proteins (for a review, see reference 10). DnaK has also been implicated in cell division (6), murein synthesis (27), and flagellar assembly (25). There are conflicting reports concerning the localization of DnaK in *Escherichia coli*. DnaK is apparently a freely soluble cytoplasmic protein, as indicated by its solubility upon bacterial lysis (29). Immunogold localization of DnaK indicates that the majority of the DnaK molecules are cytoplasmic, with the possibility that a subpopulation of DnaK is membrane associated (5). Moreover, DnaK has been found predominantly in the membrane fraction of minicells (15, 30). In the present report, we show that 40% of DnaK is released from bacterial cells by osmotic shock (20), yet all of it is retained within lysozyme-EDTA-prepared spheroplasts. Hence, DnaK belongs to the class of cytoplasmic proteins (including thioredoxin [16] and translation elongation factor Tu [EF-Tu] [13]) which are released by osmotic shock (4). It has been suggested that this reflects a localization at membrane adhesion sites where the inner and outer membranes are contiguous, as described first by Bayer (3, 12).

Bacterial strains, preparation of cell fractions, immunoblotting, and enzymatic procedures. The bacteria (*E. coli* K-12 strains C600 [*thr leu fhuA supE44 lacY*], WM1389 [C600 *dnaK756*(Ts)], BB1042 [MC4100 *thr*::Tn*10* Δ*dnaK*::Cm^r], WM1289 [C600 *dnaJ259*(Ts)], MC4100 [F$^-$ *araD139* Δ(*argF-lac*)*169 rpsL ptsF25 relA*], MM52 [MC4100 *secA51*(Ts)], CK1953 [MC4100 *secB*::Tn*5*], IQ86 [MC4100 Tn*10*], and IQ85 [IQ86 *secY24*(Ts)] were obtained from the laboratories of J. Beckwith, K. Ito, C. Kumamoto, J. C. Walker, W. Messer, and C. Georgopoulos. The strains were grown at 30°C in Luria broth (17), unless otherwise indicated, and harvested during exponential phase at a density of 5×10^8 cells per ml by centrifugation at 4°C. β-Galactosidase and galactose binding protein (MglB) were induced by 1 mM isopropyl β-D-thiogalactopyranoside and 1 mM fucose, respectively.

Osmotic shock was made as described by Nossal and Heppel (20). Unless otherwise indicated, the bacteria were plasmolyzed at 16°C in 0.03 M Tris-hydrochloride (pH 7.3)–20% (wt/vol) sucrose–0.2 mM EDTA and osmotically shocked in distilled water containing 0.3 mM $MgCl_2$ at 0°C. The shocked cells were separated from the shock fluid by centrifugation at 10,000 × *g* for 10 min. The shocked cells were lysed in 50 mM potassium phosphate, pH 6.8, by disruption in an ultrasonic disruptor (Branson Sonic Power Co.) (at a power of 25 W, five times for 15 s each time) and centrifuged at 40,000 × *g* for 10 min. The 40,000 × *g* super-natant is the shocked-cell lysate. Spheroplasts were prepared by the lysozyme-EDTA method described previously (14), and membranes were prepared by osmotic lysis of spheroplasts in the presence of 2 mM $MgCl_2$, as described by Kaback (14).

The periplasmic galactose receptor, MglB, and the DnaK protein were purified as previously described (21 and 28, respectively). The GroEL protein was purified with the help of Y. Nagata (Kyoto University) as previously described (11), and antisera against the purified proteins and against the DnaA protein were prepared in rabbits by immunization with 50 μg of protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were performed as previously described (26). Quantification of the intensity of protein bands was done in the linear range on a Helena Laboratories Quick Scan densitometer. The linear range was checked by using different amounts of purified DnaK and GroEL. The β-galactosidase assay was done as described by Miller (18); the β-galactosidase activity of membrane fractions was assayed in the presence of 0.1% Triton X-100. The assay of glucose-6-phosphate dehydrogenase activity was performed as described previously (7).

Release of DnaK by osmotic shock. The bacteria were

* Corresponding author. Mailing address: Biochimie Génètique, Institut Jacques Monod, Université Paris 7, 2 Place Jussieu, 75251 Paris Cedex 05, France. Phone: 16 1 44275098. Fax: 33 1 44273580.

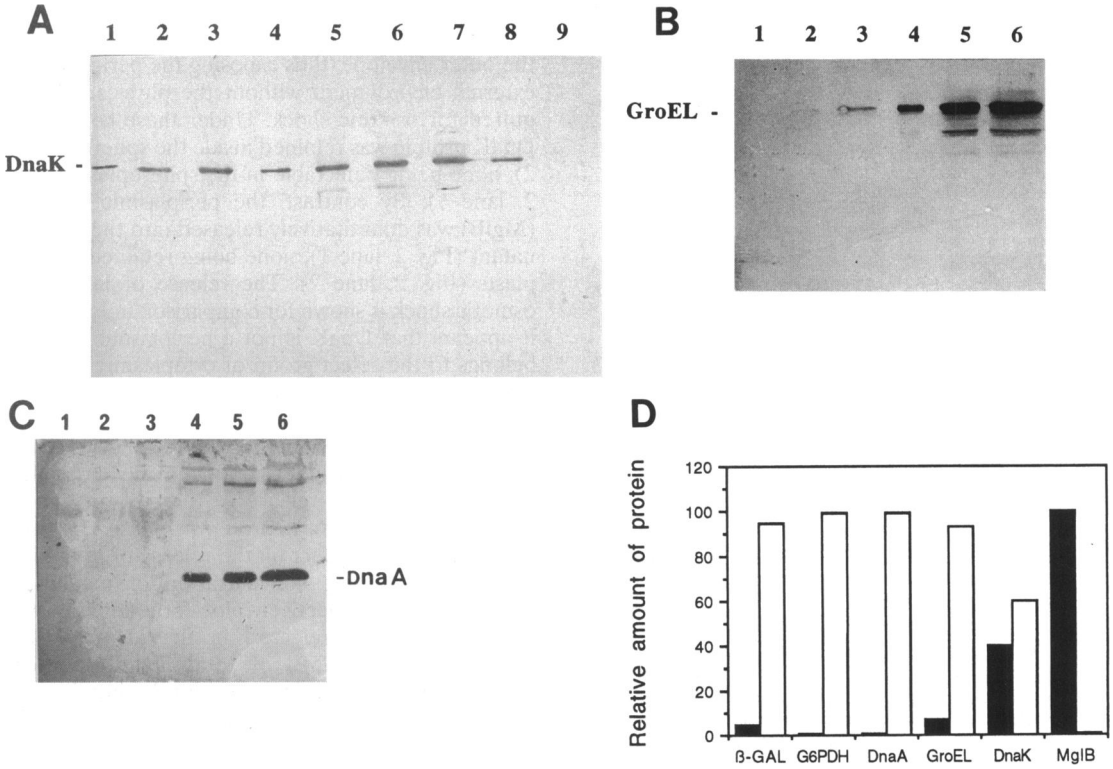

FIG. 1. Osmotic-shock release of DnaK. Cells (20 ml) at an A_{600} of 0.7 were osmotically shocked, with 2 ml of 0.3 mM $MgCl_2$ as the shock medium. Shocked cells were lysed by sonication in 2 ml of 50 mM potassium phosphate, pH 6.8. Protein concentrations in the shock fluid and in the shocked-cell lysate were 1.5 and 5.5 mg/ml, respectively. Shock fluid (6, 12, and 30 μl in lanes 1 to 3, respectively) and lysate (6, 12, and 30 μl in lanes 4 to 6, respectively) were loaded onto the gel. (A) A whole-cell pellet (equivalent to 120 μl of cells) was lysed in the electrophoresis sample buffer and loaded in lane 7 (compare lane 7 with lanes 2 and 5). An inner membrane fraction (equivalent to 120 μl of cells) was prepared as described in Materials and Methods and loaded in lane 8. A whole-cell pellet from 120 μl of the Δ*dnaK* strain was loaded in lane 9. The immunoblots were probed with anti-DnaK antibodies (A), anti-GroEL antibodies (B), and anti-DnaA antibodies (C). The relative percentages of β-galactosidase (β-GAL), glucose-6-phosphate dehydrogenase (G6PDH), DnaA, GroEL, DnaK, and MglB in the shock fluid (solid bars) and in the shocked-cell lysate (open bars) were calculated from three independent experiments and are shown in panel D. β-Galactosidase and glucose-6-phosphate dehydrogenase were assayed as described in Materials and Methods, and relative DnaA, DnaK, GroEL, and MglB concentrations were quantified from immunoblots as described in Materials and Methods.

osmotically shocked according to the procedure of Nossal and Heppel (20), which quantitatively releases most of the periplasmic proteins while most of the cytoplasmic proteins remain inside the cells. Using antibody detection or enzyme activity assays, we analyzed the shock fluid and the shocked-cell lysate for the presence of five cytoplasmic proteins: DnaK/hsp70, GroEL/hsp60, DnaA (replication initiator), β-galactosidase, and glucose-6-phosphate dehydrogenase. Approximately 40% of soluble DnaK is released by osmotic shock (Fig. 1A, lanes 1 to 3), 60% being found in the shocked-cell lysate (lanes 4 to 6). The total amount of DnaK (100%) in whole cells is shown in Fig. 1A, lane 7 (this extract contains the same amount of cells as lanes 2, 5, and 8). Twenty percent of total DnaK is found in membrane fractions (Fig. 1A, lane 8, containing the same amount of cells as lanes 2, 5, and 7) (see below). The Δ*dnaK* strain BB1042 does not react with anti-DnaK antibodies (Fig. 1A, lane 9).

In contrast to DnaK, only small amounts of GroEL (7%) (Fig. 1B, lanes 1 to 3), DnaA (less than 1%) (Fig. 1C, lanes 1 to 3), β-galactosidase (less than 5%) (Fig. 1D), and glucose-6-phosphate dehydrogenase (less than 1%) (Fig. 1D) are released into the shock fluid. These cytoplasmic proteins are mostly retained inside the cells (Fig. 1B and C, lanes 4 to 6, and Fig. 1D). The retention of four cytoplasmic proteins (GroEL,

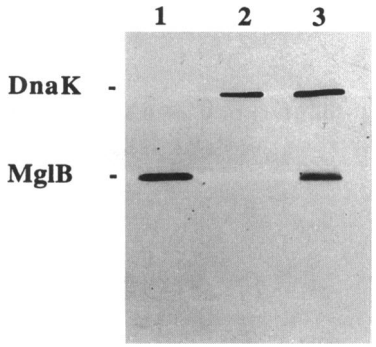

FIG. 2. Retention of DnaK in spheroplasts. Lysozyme-EDTA-prepared spheroplasts were prepared as described in Materials and Methods, producing 4 ml of spheroplast supernatant (0.7 mg of protein per ml); subsequently, the spheroplasts were lysed by sonication in 2 ml of potassium phosphate, pH 6.8 (6 mg of protein per ml). Spheroplast supernatant (20 μl) and spheroplast lysate (4 μl) were loaded in lanes 1 and 2, respectively. Shock fluid (5 μl) prepared as described in the legend to Fig. 1, was loaded in lane 3. The immunoblots were visualized with anti-DnaK and anti-MglB antibodies.

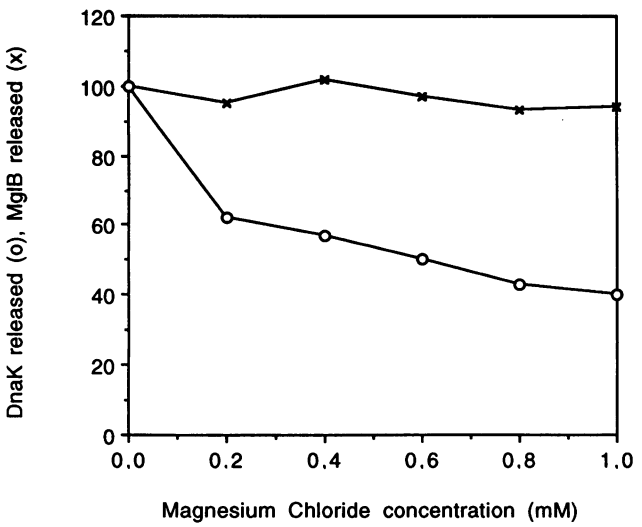

FIG. 3. Effect of $MgCl_2$ on the release of DnaK by osmotic shock. Cells were plasmolyzed in sucrose-EDTA and then diluted into distilled water containing magnesium chloride at the indicated concentrations. Protein levels were determined by densitometric scanning of immunoblots, as described in Materials and Methods. The percentage of protein released by osmotic shock was determined relative to the amount released in the absence of magnesium chloride. The maximal release of MglB represents 100% of total MglB, and the maximal release of DnaK represents 55% of total DnaK. Less than 6% of β-galactosidase was released at all magnesium chloride concentrations.

DnaA, β-galactosidase, and glucose-6-phosphate dehydrogenase) inside shocked cells suggests that there is not extensive cell damage during the osmotic-shock treatment and contrasts sharply with the release of DnaK by the osmotic-shock procedure (Fig. 1D). As expected, the osmotic shock quantitatively releases the periplasmic galactose receptor (MglB) for transport and chemotaxis (Fig. 1D).

Retention of DnaK inside spheroplasts. To differentiate between periplasmic and cytosolic localization, the release of DnaK was examined after the formation of lysozyme-EDTA-prepared spheroplasts. Lysozyme-EDTA treatment disrupts the outer envelope, thus exposing the periplasmic space to the external environment without the physical manipulations required for osmotic shock. Under these conditions, all of the DnaK protein was retained inside the spheroplasts (Fig. 2, lane 2), none being detectable in the spheroplast supernatant (Fig. 2, lane 1). By contrast, the periplasmic galactose receptor (MglB) was quantitatively released into the spheroplast supernatant (Fig. 2, lane 1), none being retained inside the spheroplasts (Fig. 2, lane 2). The release of DnaK and MglB by osmotic shock is shown for comparison in Fig. 2, lane 3. Hence, it appears that DnaK is not a periplasmic protein but rather belongs to the select group of cytoplasmic proteins which are released by osmotic shock, which also includes thioredoxin, EF-Tu, and penicillin-binding protein 1b (16).

$MgCl_2$ dependence of the osmotic-shock release of DnaK. The presence of divalent cations in the shock fluid has been shown to block the release of thioredoxin and translational EF-Tu (two cytoplasmic proteins released by osmotic shock) while having little effect on the release of periplasmic proteins (16). An increasing concentration of magnesium chloride during the shock selectively blocks the release of DnaK (55% of total DnaK is released in the absence of magnesium chloride, whereas 25% of total DnaK is released in the presence of 1 mM magnesium chloride) but does not affect the release of the periplasmic galactose receptor MglB (Fig. 3). This result provides further evidence that DnaK is a cytoplasmic protein that is released by osmotic shock.

Possible peripheral membrane association. It has been suggested that the release of cytoplasmic proteins by osmotic shock requires a peripheral membrane association at the inner surface of the cytoplasmic membrane (4). Such an association was observed for thioredoxin (16) when cells were lysed under gentle conditions. The association of DnaK with membrane fractions has also been reported previously (15, 30). When membranes are prepared by the gentle lysis technique of Kaback (14), 20% of DnaK is recovered in the membrane pellet (Fig. 1A, lane 8), compared with 6% of β-galactosidase (not shown), suggesting that a fraction of DnaK is associated with the inner membrane.

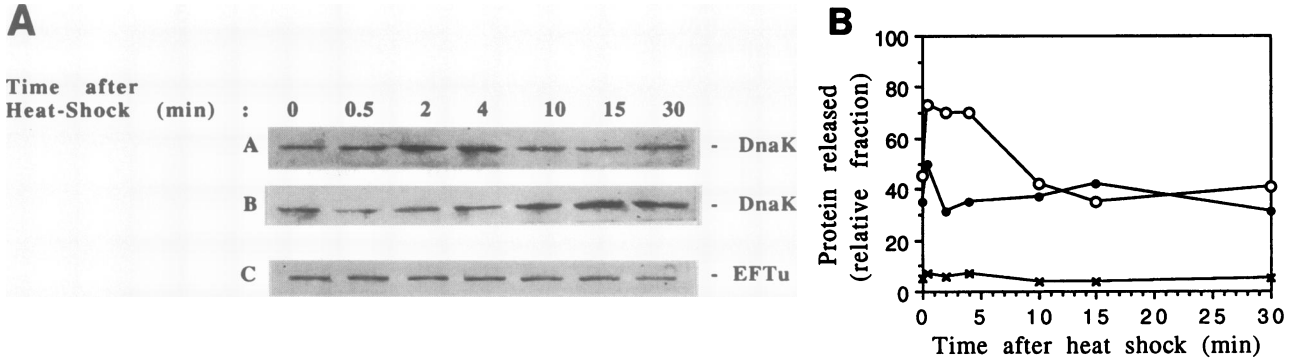

FIG. 4. Osmotic-shock release of DnaK after heat shock. The bacteria were subjected to a heat shock treatment from 30 to 42°C. They were transferred to ice at the indicated times and osmotically shocked at 0°C, as described in Materials and Methods and in the legend to Fig. 1. All steps were carried out at 0°C, and the whole procedure lasted less than 15 min. (A) Shock fluid (10 μl) (lane A) and shocked-cell lysate (10 μl) (lane B) were loaded onto the gel and blotted with anti-DnaK antibodies. Shock fluid (10 μl) (lane C) was blotted with anti-EF-Tu antibodies. (B) The amount of β-galactosidase in the shock fluid was measured as described in Materials and Methods. The relative amounts of DnaK (○), EF-Tu (●), and β-galactosidase (×) released in the shock fluid are expressed as follows: amount of protein released in the shock fluid/(amount of protein released + amount of protein retained in the shocked-cell lysate) × 100. DnaK and EF-Tu levels (mean values of three independent experiments) were determined by densitometric scanning of immunoblots, as described in Materials and Methods.

Osmotic-shock release of DnaK during heat shock. The bacteria were subjected to a heat shock (from 30 to 42°C) and then transferred to ice at various times after this treatment. The release of DnaK in the shock fluid increased during the first 10 min after heat shock (Fig. 4A, lanes A [DnaK released] and B [DnaK retained inside shocked cells]). However, there was no significant increase in the release of EF-Tu (Fig. 4A, lane C) or β-galactosidase (Fig. 4B). No significant increase in the osmotic-shock release of DnaK was observed when cells grown at 42°C (without heat shock) were used for the osmotic-shock treatment or when cells grown at 25°C were transferred to 37°C (data not shown). Hence, it seems that there is an increased localization of DnaK in the osmotic-shock-sensitive compartment of the cytoplasm after a heat shock. Heat shock-induced changes in the localization of several heat shock proteins has been observed for other systems (10).

Osmotic-shock release of DnaK in *secB*, *secA*, *secY*, *dnaJ*, and *dnaK* mutants. Since DnaK has been implicated in protein export (for a review, see reference 9), the osmotic-shock release of DnaK was measured in three mutants of the protein membrane translocator (24) (*secB*::Tn*5*, *secA51*, and *secY24*) and found to be similar to that of the parental strains (data not shown). The release of DnaK is also not affected in the *dnaJ259* mutant (data not shown). However, the *dnaK* thermosensitive mutant *dnaK756* releases a decreased amount of DnaK (60% of that released by the parental strain) (data not shown), a result which is consistent with a potential physiological function associated with the osmotic-shock release of the DnaK chaperone.

Implications. We show in this report that DnaK behaves in the same way as a small number of cytoplasmic proteins including thioredoxin and EF-Tu (13, 16), in that it is released by osmotic shock but retained in lysozyme-EDTA-prepared spheroplasts. It has been suggested that cytoplasmic proteins released by osmotic shock are associated with the inner membrane and that they pass by extrusion through regions in which the inner and outer membranes are contiguous (2). Indeed, a partial association of thioredoxin with Bayer adhesion sites has been demonstrated by immunoelectron microscopy (2), and this localization has been related to the involvement of the protein in the assembly of filamentous phages which occurs at such sites (23) or in the redox control of disulfide bonds in polypeptides exported from the cell (19). It has been suggested that DnaK is implicated in protein export (for a review, see reference 9), bacterial cell division (6) murein synthesis (27), and the assembly of flagellar components (25). These functions might require membrane association or localization of DnaK at membrane adhesion sites. Furthermore, the DnaJ protein, which functions in association with DnaK, seems to possess a membrane localization (10). Finally, it might be postulated that DnaK participates in some as-yet-undefined membrane function. The hydrophobic binding site of this chaperone, which facilitates its interaction with hydrophobic regions of other proteins (22), might also permit it to interact with the hydrophobic regions of molecules such as phospholipids or lipopolysaccharides.

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