73-kDa Molecular Chaperone HSP73 Is a Direct Target of Antibiotic Gentamicin*

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Toshibo Miyazaki†, Ryo Sagawa†, Takenori Honma†, Susumu Noguchi†, Taisuke Harada†, Atsushi Komatsuda†, Hiroshi Ohtani†, Hideki Wakuš†, Ken-ichi Sawada†, Michio Otaka†, Sumio Watanabe‡, Mitsutoshi Jikei‡, Nobuaki Ogawa‡, Funio Hamada‡, and Hideaki Itoh‡

From the †Department of Material-process Engineering and Applied Chemistry for Environment, Akita University Faculty of Engineering and Resource Science, 1-1 Tegata Gakuen Town, Akita City 010-8502, Japan and ‡Third Department of Internal Medicine, ‡First Department of Internal Medicine, Akita University School of Medicine, 1-1-1 Hondo, Akita City 010-8543, Japan

Although gentamicin (GM) has been used widely as an antibiotic, the specific binding protein of the drug has not yet been understood sufficiently. Here we show that GM specifically associates with the 73-kDa molecular chaperone HSP73 and reduces its chaperone activity in vitro. In the present study, we investigated GM-specific binding proteins using a GM-affinity column and porcine kidney cytosol. After washing the column, only the 73-kDa protein was eluted from the column by the addition of 10 mM GM. None of the other proteins were found in the eluant. Upon immunoblotting, the protein was identical to HSP73. Upon CD spectrum analysis, the binding of GM to HSP73 resulted in a conformational change in the protein. Although HSP73 prevents aggregation of unfolded rhodanese in vitro, the chaperone activity of HSP73 was suppressed in the presence of GM. Using limited proteolysis of HSP73 by TPCK-trypsin, the GM binding site is a COOH-terminal for one third of the protein known to be a peptide-binding domain. During immunohistochemistry, HSP73 and GM were co-localized in enlarged lysosomes of rat kidneys with GM-induced acute tubular injury in vivo. Our results suggest that the specific association between HSP73 and GM may reduce the chaperone activity of HSP73 in vitro and in vivo, and this may have an interaction with GM toxicity in kidneys with GM-induced acute tubular injury.

Until about 10 years ago, it had been assumed that the folding of newly synthesized polypeptides in vivo occurs spontaneously without assisting any other proteins or metabolic energy, because unfolded proteins can reach their native conformation (their specific tertiary structure) spontaneously in vitro (1). Now it is thought that the folding of many newly synthesized polypeptides in the cell are assisted by a class of proteins (molecular chaperones) which function mainly in preventing off-pathway folding reactions that lead to aggregation. Heat shock proteins (HSPs)1, also called molecular chaperones, are highly conserved proteins and are rapidly induced in cells in response to abrupt and adverse changes in their environment (2–4). These HSPs are constitutively expressed and fulfill functions as “molecular chaperones” under normal cellular conditions. Mammalian HSPs are classified into four families according to their approximate molecular masses and the degree of homology (2, 3).

The cytosolic 70-kDa molecular chaperones (HSP70s) are present in cells as two different gene products but are closely related to each other: a stress-inducible form, HSP72 (known as HSP70), and a constitutively expressed form, HSP73 (known as 70-kDa heat shock cognate protein, HSC70) (3). HSP73 consists of two domains, a highly conserved NH2-terminal ATPase domain, with a molecular mass of 45 kDa and a COOH-terminal domain of 25 kDa (5, 6). An 18-kDa protein of the COOH-terminal adjacent to the ATPase domain contains the polypeptide-binding site (7, 8). The ATP-bound form of HSP70 binds and releases peptide rapidly, whereas the ADP form binds peptide slowly but more stably (9, 10). Binding ATP to the ATP-binding domain causes a conformational change, which in turn results in structural alterations in the COOH-terminal domain, thus leading to substrate release (11–13). HSP70s and their partner proteins can stabilize newly synthesized polypeptides until all segments of the chain necessary for folding are available.

We have reported previously that the induction of HSP73 in injured tubular epithelial rat kidneys with gentamicin (GM)-induced acute renal failure (14). GM is widely used as a bactericidal agent for the treatment of severe Gram-negative infections; however, its clinical use is partially limited because of its nephrotoxicity. Recent evidence suggests a role of reactive oxygen metabolites in GM nephropathy (15). There are many reports about the clinical effects of GM, but no reports have been made for GM-specific binding proteins. In the present study, we investigated binding proteins to GM using GM-affinity column chromatography and identified a protein with a molecular mass of 73 kDa. We will discuss its physiological meanings and its possible functions, especially in kidneys with GM-induced acute tubular injury.

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1 The abbreviations used are: HSP, heat shock protein; HSP73 and HSP72, heat shock proteins with subunit molecular masses of 73 and 72 kDa, respectively; GM, gentamicin; NAG, N-acetyl-β-D-glucosaminidase; CD, circular dichroism; TPCK-trypsin, treated with tosylphenylalanin chloromethane trypsin.

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To whom correspondence should be addressed: Dept. of Material-process Engineering and Applied Chemistry for Environment, Akita University Faculty of Engineering and Resource Science, 1-1 Tegata Gakuen Town, Akita City 010-8502, Japan. Tel.: 81-18-889-3041; Fax: 81-18-883-3041; E-mail: itohh@ipc.akita-u.ac.jp.
HSP73 Binds to Gentamicin

**MATERIALS AND METHODS**

**GM-affinity Column Chromatography**—GM-Sepharose was prepared using GM (Sigma) and CH-activated Sepharose 4B (Amersham Biosciences) according to the manufacturer’s instructions. Bovine HSP73 (ATP- or ADP-bound form) was prepared from brain using ATP-Sepharose, as described previously; an anti-HSP73 antibody against bovine brain HSP73 was produced as described previously (16). An antibody against bovine HSP72 was produced as described previously (16). Bovine HSP73 (ATP-free form) was purified from brain using an ammonium-sulfate fractionation, DEAE-Sepharose, and hydroxyapatite column chromatography. At each step, HSP73 was detected with immunoblotting by using an anti-HSP73. Porcine kidney cytosols in 10 mM Tris-HCl (pH 7.4) were applied to the column equilibrated with the same buffer and washed with 20 column volumes of the buffer containing 0.15 mM NaCl. After washing the column extensively, binding proteins were eluted with 10 mM GM in the same buffer or with a linear gradient of GM (0–10 mM). The eluants were analyzed on SDS/PAGE (17) or by immunoblotting (18) using an anti-HSP73 or -HSP72 antibody. The eluants and purified HSP73 were analyzed by two-dimensional gel electrophoresis (19). Samples were electrophoresed in the first-dimension on an isoelectric focusing gel with a pH gradient of 5–7. Electrophoresis for the second dimension was performed on a 7% acrylamide slab gel with SDS. After electrophoresis, gels were stained with 0.1% Coomassie Brilliant Blue R-250 in a mixture of 25% isopropyl alcohol and 10% acetic acid; they were destained with 10% isopropyl alcohol and 10% acetic acid or by immunoblotting (18) using an anti-HSP73 or -HSP72 antibody and alkaline phosphatase anti-rabbit IgG antibody (Sigma). In this study, we used CH-activated Sepharose 4B as a GM-Sepharose column and were eluted with a linear gradient of GM (0–5 mM in the buffer). The eluants were electrophoresed on SDS/PAGE and stained with Coomassie Brilliant Blue.

**Far-UV Circular Dichroism (CD)—CD measurements were performed on a J-720 spectropolarimeter (Jasco) as described previously (20, 21).** 50 mM HEPES buffer (pH 7.4) in the presence or absence of GM (1 mM) was used as a blank. Purified HSP73 (1.5 mM) was included in 50 mM HEPES buffer (pH 7.4) in the presence or absence of GM (1 mM) was filtered through a 0.22-μm filter, and scans were carried out at 25 °C in the 240- and 200-nm regions in a cuvette with a 0.5-mm path length. The observed specific ellipticity (difference between sample and blank) was converted to the mean residue ellipticity Φ (degrees/cm²/dmol).

**Measurement of Protein Aggregation**—The influence of GM on the chaperone activity of HSP73 was monitored using rhodanese. Rhodanese (Sigma) was denatured at a concentration of 100 μM in buffer containing 6 mM guanidinium chloride (GdmCl), 25 mM HEPES-KOH, pH 7.5. Denatured rhodanese was rapidly denatured to 0.5 μM in buffer containing 25 mM HEPES-KOH, pH 7.5, in the presence or absence of HSP73 (250 μM) and GM (500 μM). Aggregation of rhodanese was monitored over 10 min at A230 in an Amersham Biosciences Ultrospec 3000 UV-Vis spectrophotometer, and the data were normalized using the aggregation in buffer alone as the standard.

**GM-binding Sites of HSP73—**Purified HSP73 (ATP-free form, 5 mg/ml) was mildly digested by 0.5 μg/ml TPCK-trypsin (Roche Applied Science) at 37 °C for 60 min. The TPCK-trypsin digests were applied to a GM-Sepharose column and were eluted as described above. After detection of the eluants on SDS/12.5% PAGE, the 45-, 32-, and 29-kDa peptide bands were excised and digested using lysyl endopeptidase (Wako) as described previously (22, 23). The peptides were purified by the reverse-phase HPLC (Wakoil SC18), which was connected to a high-performance liquid chromatography apparatus (Jasco PU-1580 pump and Amersham Biosciences 2158 Uvicord S.D. UV detector). Peptides were purified from the column with a linear gradient of 0–60% acetonitrile at a flow rate of 1.0 mL/min, and 0.5-ml fractions were collected as described previously (22, 23). The amino acid sequence of the purified peptides was determined with a 491 Procise protein sequence system (PerkinElmer Life Sciences).

**Animal Model for Acute Gentamicin Nephropathy**—The protocols for animal experimentation described here were approved by the Animal Research Committee, Akita University School of Medicine. All subsequent animal experiments adhered to the “Guidelines for Animal Experimentation” of the University. Essential protocols for gentamicin-induced acute renal failure have been described previously (14). Male Sprague-Dawley rats were administered intramuscular injections of gentamicin (80 mg/kg of body weight), and control rats were injected physiologic saline once a day for 14 days. Kidneys from these rats were obtained at 0, 24, and 36 h, and days 3, 6, 9, 12, 15, 18, 21, 24, and 27. Urine was collected from each rat at the above time points, and urinary volume and N-acetyl-β-D-glucosaminidase (NAG) levels were measured. Blood was also taken from the subclavian vein of each rat immediately after a urine collection at the above time points. Blood urea nitrogen and serum creatinine were measured.

Renal immunohistochemical localization of HSP73 was serially examined. In addition, kidneys were homogenized with isotonic buffer (10 mM Tris-HCl, pH 7.4, containing 0.15 mM NaCl) and centrifuged at 20,000 × g for 20 min at 4 °C. The supernatants were used as soluble fractions. The precipitates were washed with the same buffer for three times and the precipitates were resuspended with 8 mM urea, followed by centrifugation at 20,000 × g for 20 min at 4 °C. The supernatants were collected and used as insoluble fractions. HSP73 in both soluble and insoluble fractions was measured by immunoblot analysis (18).

**Electron Microscopic Immunohistochemistry**—Electron microscopic immunohistochemistry of rat kidneys was performed as described previously (22). Briefly, ultrathin sections of rat kidneys were cut with a diamond knife and mounted on gold grids. The sections were stained by the immunogold silver-staining method for electron microscopy, using a silver enhancing kit (BioCell Research Laboratories). These sections were incubated with mouse monoclonal antibody against GM (O.E.M. Concepts, Inc.) or rabbit polyclonal antibodies against HSP73 (200-fold dilution in buffer E. 10 mM phosphate buffer, pH 7.4, containing 2% NaCl, 0.1% Tween 20) for 18 h at room temperature. The sections were washed with buffer E and incubated with gold-labeled anti-rabbit IgG or gold-labeled anti-mouse IgG (Nanoprobes; 400-fold dilution in buffer E) for 1 h at room temperature. After further washing with distilled water, the sections were incubated with the silver developer of the

![Fig. 1. GM affinity-column chromatography. Porcine kidney cytosols were applied to GM-affinity column, washed with 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 mM NaCl, and proteins were eluted with 10 mM GM or a linear gradient of 0–10 mM GM. The eluants with 10 mM GM were analyzed on SDS/PAGE, followed by staining with Coomassie Brilliant Blue (A) or immunoblotting using an anti-HSP73 antibody or an anti-HSP72 antibody (D). In both panels (A and D), S, W, and E denote applied samples, washed with buffer containing 0.15 mM NaCl, and eluted proteins, respectively. C, the eluants with 10 mM GM were also analyzed on two-dimensional gel electrophoresis, followed by staining with Coomassie Brilliant Blue (left), immunoblotting using an anti-HSP72 antibody (middle), or an anti-HSP73 antibody (right). E, the eluants with linear gradient of 0–10 mM GM were electrophoresed on SDS/PAGE, followed by staining with Coomassie Brilliant Blue. S, P, and W denote applied samples, pass-through fraction, and washed proteins, respectively. B, purified bovine HSP73 was analyzed on two-dimensional gel electrophoresis, followed by staining with Coomassie Brilliant Blue (left) or immunoblotting using an anti-HSP72 antibody (middle) or an anti-HSP73 antibody (right). Left panel, arrow and arrowhead indicate HSP73 and HSP72, respectively.](http://www.jbc.org/)

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RESULTS

GM Affinity-column Chromatography—To avoid nonspecific binding proteins, GM-affinity columns were thoroughly washed with a buffer containing 0.15 M NaCl. Proteins were eluted with 10 mM GM from those affinity columns and the eluants were detected on SDS/PAGE or two-dimensional gel electrophoresis (Fig. 1). Although no protein bands were detected in the eluants from the control column (data not shown), protein bands with molecular masses of ~70 kDa were detected in the eluant (Fig. 1A).

In the present study, we used two different antibodies against HSP73 or HSP72. The purified bovine HSP73 was separated into two spots (73 and 72 kDa) on two-dimensional gel electrophoresis (Fig. 1B, left). The amount of HSP73 was much greater than that of HSP72. The isoelectric points of HSP73 and HSP72 were 5.7 and 5.8, respectively. An antibody against HSP72 or HSP73 was cross-reacted to the purified HSP72 and HSP73, respectively (Fig. 1B, middle and right panels). These antibodies can recognize HSP72 and HSP73 with great sensitivity. Only one spot was detected in the eluants from GM-affinity column on two-dimensional gel electrophoresis (Fig. 1B). Although no protein bands were detected in the eluant (Fig. 1C, left), no protein spots were detected in the eluant by immunoblotting with an anti-HSP72 antibody (Fig. 1C, middle). On the contrary, we did detect one spot in the eluants by immunoblotting with an anti-HSP73 antibody (Fig. 1C, right). The protein was identical to HSP73 by immunoblotting with an anti-HSP73 antibody (Fig. 1D). To confirm the results, we analyzed the elution pattern of HSP73 from the affinity column with a linear gradient of GM (Fig. 1E). With SDS/PAGE, we could find no other proteins except for HSP73 in the eluants at the given conditions. These results suggest that HSP73 is a direct target of the antibiotic gentamicin.

Conformational Change in HSP73 upon Binding with GM—It has been reported that HSP73 has an average composition of 40% α-helix and 20% periodic structure within HSP73 and between 16–41% β-sheet and 21–0% β-turn (24). Based on the available information, we analyzed whether GM may give rise to a conformational change (secondary structure) in HSP73 using a far-UV CD spectrum. As shown in Fig. 2A, our data (HSP73 alone) were in good agreement with the CD spectrum data of HSP73 (24). The binding of GM to HSP73 resulted in the conformational changes in HSP73. By binding GM to HSP73, decreasing the mean residue ellipticity θ at or near 208 nm and increasing θ at or near 218 nm indicated the decreasing α-helix and increasing β-strand structure of HSP73, respectively, in the CD spectrum (Fig. 2A). These results indicate that the binding of GM to HSP73 is not a transient association and may cause a conformational change in the protein with a significant meaning.

Measurement of Protein Aggregation—The chaperone activity of HSP73 can be detected by the ability of purified HSP73 to prevent aggregation of unfolded polypeptide substrate such as...
rhodanese (25). The protein has a pronounced tendency toward aggregation upon dilution of the denaturant GdmCl into the physiological buffer solution. In the present study, we analyzed the chaperone activity of HSP73 by using this system. HSP73 was capable of preventing the aggregation of rhodanese (Fig. 2B). The chaperone activity of HSP73 was reduced almost completely in the presence of a molar ratio of GM:HSP73 of 2:1. GM alone did not have an effect upon the aggregation of unfolded rhodanese. The results suggest that HSP73 has chaperone activity against unfolded polypeptide, but the ability is reduced by GM.

**GM-binding Sites of HSP73**—If the GM-binding site is the same as the ATP-binding domain of HSP73, GM will be unable to bind to the ATP-bound form of HSP73. For these reasons, we used the ATP-free form of HSP73 in this study. As shown in Fig. 3A, HSP73 was mildly digested by TPCK-trypsin. Digests with molecular masses of 45-, 32-, and 29-kDa were applied to the GM-affinity column and eluted with a linear gradient of GM (0–5 mM). Interestingly, the 45-kDa fragment was unable to bind to the column, and the 29-kDa peptide was detected in the eluant at the end of the linear gradient of GM (5 mM) to some extent. In these peptides, the 29-kDa peptide had the highest affinity to GM compared with the others. To analyze the amino acid sequence of the TPCK-trypsin digests, the 45-, 32-, and 29-kDa peptides were excised from gel and digested by lysyl endopeptidase. Three peptides of the 45-kDa fragment were separated and sequenced. As shown in Fig. 3B, the 45-kDa fragment was NH2-terminal of HSP73, which is known to be an ATP-binding domain. One peptide of the 32-kDa fragment and two peptides of the 29-kDa fragment were located in the COOH-terminal of HSP73. As mentioned above, the 29-kDa fragment, rather than the other two fragments, was eluted later in a linear gradient of GM. These results suggest that GM binds to the COOH-terminal one-third of HSP73. The linear structure of rat HSP73 is shown in Fig. 3C.

**GM-treated Rats**—Gentamicin associates to HSP73 in vitro. We analyzed the association also in vivo. The levels of urinary volume, NAG, blood urea nitrogen, and serum creatinine in gentamicin-treated rats are shown in Fig. 4. The urinary volume in GM-treated rats began to increase on day 6, reached a peak level on day 15, and decreased on day 21. Urinary NAG levels also increased on day 6, reached a peak level on day 12, and decreased on day 15. The profile levels of both blood urea nitrogen and serum creatinine were similarly parallel with those of NAG.
GM in the GM-treated rat kidneys. These results suggest that GM binds to HSP73 not only in vitro but also in vivo.

**DISCUSSION**

Although the antibiotic gentamicin has been widely used clinically, its use is partially limited because of its renal toxicity (27). Gentamicin has been shown to enhance the generation of superoxide anion and hydrogen peroxide by renal cortical mitochondria (15). It has been assumed that GM inhibits the activity of lysosomal phospholipase in proximal tubular epithelial cells (28). Because of the GM nephrotoxicity, tubular cells are damaged and phospholipidosis occurs in the damaged tubular cells. The injured lysosomes contain accumulated phospholipids, the so-called myeloid bodies. In our previous study, HSP73 molecular chaperones were rapidly induced in rat kidneys with GM-induced acute tubular injury (14). The physiological meanings of the induction of these molecular chaperones in injured rat kidneys by GM have not yet been fully understood. To investigate the toxicity or the induction of HSP73 by GM, it is necessary to know the specific binding proteins to GM in vitro and in vivo. Until now, there have been no reports for specific binding proteins to GM. In the present study, we investigated the possibility of direct interactions of GM to molecular chaperones and the effect upon their chaperone activity by GM.

70-kDa molecular chaperones (HSP70s) are a family of highly conserved protein, and they have essential roles in protein metabolism under both stress and normal conditions, including functions in de novo protein folding, membrane translocation, the degradation of misfolded proteins, as well as regulatory process (2, 3). The HSP70s are subdivided into an ATPase domain of 45 kDa and followed by a 18-kDa domain containing the peptide binding site (7, 8). It has been shown that the peptide-binding domain of HSP73 contains two four-stranded antiparallel β-sheets and a single α-helix by multidimensional NMR analysis (7). As shown in Fig. 3C, the COOH-
terminal peptide-binding domain (amino acids 384–612) is composed of a β-sandwich and an α-helical subdomain (13). The COOH-terminal of about 30 amino acids is composed of a G/P-rich region and ends with a conserved EEDV motif (13). In the present study, the binding of GM to HSP73 resulted in a conformational change in the protein in CD spectrum analysis. By binding GM to the protein, a portion of α-helix was decreased, whereas a portion of β-sheets was increased. On the analysis binding site of GM to HSP73 using trypsin digests of the protein, the 29-kDa fragment has a high affinity to GM and the fragment was located in the COOH-terminal one-third of HSP73. By binding GM to the peptide-binding domain, the result may be the decrease of α-helix and the increase of β-sheets in the peptide-binding domain. These results suggest that GM binds to the COOH-terminal of HSP73, the so-called peptide-binding domain, leading to the conformational change in the molecular chaperone.

The various HSP70s all have binding specificity for hydrophobic peptides, but the presence of certain amino acid patterns may vary from compartment to compartment (28, 29). The peptides are bound in an extended conformation and a preferred pattern of certain aliphatic and aromatic residues at alternating positions (12). HSP73 has four putative binding pockets for alternating amino acid residues in the peptide-binding domain: large hydrophobic residues, including both aromatic (W, F) and aliphatic (L) residues but not Y, which has a polar moiety at the end of side chain; large hydrophobic residues including both aromatic (W, F, Y) and aliphatic (L, I) residues, but not Y, which cause nephrotoxicity by GM in the kidney.

Based upon the present study, GM may be able to bind to the peptide binding domain of HSP73. Under the conditions of this study, many unfolded proteins that were in need of folding by HSP73 were unable to bind to the molecular chaperone. The accumulation of unfolded proteins and GM, which is bound and protected by HSP73 in the lysosomes in proximal tubules, may cause nephrotoxicity by GM in the kidney.

In our present study, GM preferentially binds to the peptide binding domain of HSP73. Under conditions of this study, many unfolded proteins that were in need of folding by HSP73 were unable to bind to the molecular chaperone. The accumulation of unfolded proteins and GM, which is bound and protected by HSP73 in the lysosomes in proximal tubules, may cause nephrotoxicity by GM in the kidney.

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Toshio Miyazaki, Ryo Sagawa, Takenori Honma, Susumu Noguchi, Taisuke Harada,
Atsushi Komatsuda, Hiroshi Ohtani, Hideki Wakui, Ken-ichi Sawada, Michiro Otaka,
Sumio Watanabe, Mitsutoshi Jikei, Nobuaki Ogawa, Fumio Hamada and Hideaki Itoh

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