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The Role of the Cysteine Residue in the Chaperone and Anti-Apoptotic Functions of Human Hsp27

NagaRekha Pasupuleti¹, Mahesha Gangadhariah¹, Smitha Padmanabha¹, Puttur Santhoshkumar², and Ram H. Nagaraj^{1,3,*}

¹Department of Ophthalmology & Visual sciences, Case Western Reserve University, Cleveland, Ohio 44106

²Department of Ophthalmology, University of Missouri, Columbia, Missouri 65211

³Department of Pharmacology, Case Western Reserve University, Cleveland, Ohio 44106

Abstract

The small heat shock protein Hsp27 is a molecular chaperone and an anti-apoptotic protein. Human Hsp27 has one cysteine residue at position 137. We investigated the role of this cysteine residue in the chaperone and anti-apoptotic functions of Hsp27 by mutating the cysteine residue to an alanine (Hsp27_{C137A}) and comparing it to wild-type protein (Hsp27_{WT}). Both proteins were multi-subunit oligomers, but subunits of Hsp27_{WT} were disulfide-linked unlike those of Hsp27_{C137A}, which were monomeric. Hsp27_{C137A} was indistinguishable from Hsp27_{WT} with regard to its secondary structure, surface hydrophobicity, oligomeric size and chaperone function. S-thiolation and reductive methylation of the cysteine residue had no apparent effect on the chaperone function of Hsp27_{WT}. The anti-apoptotic function of Hsp27_{C137A} and Hsp27_{WT} was studied by overexpressing them in CHO cells. No difference in the caspase-3 or -9 activity was observed in staurosporine-treated cells. The rate of apoptosis between Hsp27_{C137A} and Hsp27_{WT} overexpressing cells was similar whether the cells were treated with staurosporine or etoposide. However, the mutant protein was less protective relative to the wild-type protein in preventing caspase-3 and caspase-9 activation and apoptosis induced by 1 mM H₂O₂ in CHO and HeLa cells. These data demonstrate that in human Hsp27, disulfide formation by the lone cysteine does not affect its chaperone function and anti-apoptotic function against chemical toxicants. However, oxidation of the lone cysteine in Hsp27 might at least partially affect the anti-apoptotic function against oxidative stress.

Keywords

Hsp27; CHAPERONE; APOPTOSIS; CYSTEINE

Hsp27 belongs to the small heat shock protein family. This family is comprised of more than 10 members, all of which have the core “ α -crystallin” domain, which is flanked by a short hydrophilic carboxyl terminus and a hydrophobic amino terminus. Hsp27 is found in

*Correspondence to: Prof. Ram H. Nagaraj, PhD, Department of Ophthalmology & Visual Sciences, Case Western Reserve University, Cleveland, OH 44106. ram.nagaraj@case.edu.

oligomeric state in cells. The oligomeric state is polydisperse, and it can range from 54 to 700 kDa [Lambert et al., 1999]. The size of the oligomer is reduced by phosphorylation. Hsp27 has three serine residues at 15, 78, and 82 that are phosphorylated by MAPKAP kinase-2 [Stokoe et al., 1992] or protein kinase-D [Yuan and Rozengurt, 2008]. Phosphorylation at the three sites results in the conversion of Hsp27 to smaller tetrameric aggregates [Rogalla et al., 1999].

Hsp27 is a molecular chaperone. It binds to structurally perturbed client proteins and prevents their denaturation. Unlike other stress proteins, such as Hsp90 and Hsp70, Hsp27 does not require ATP for this function [Jakob et al., 1993]. Once Hsp27 binds and stabilizes the client protein, other stress proteins release the client proteins from the Hsp27 complex so they can recover. In stressed cells, Hsp27 undergoes phosphorylation [Chretien and Landry, 1988; Barchowsky et al., 1994]. Whether phosphorylation affects the chaperone function is still being debated. Recent studies suggest that phosphorylation improves the chaperone function [Stege et al., 1995; Shashidharamurthy et al., 2005; Hayes et al., 2009], while other studies have shown the contrary [Mehlen et al., 1996; Rogalla et al., 1999; Oya-Ito et al., 2006]. In addition, Hsp27 regulates intracellular redox status by upregulating GSH levels [Mehlen et al., 1997].

Hsp27 is also an anti-apoptotic protein [Garrido et al., 2001]. Cells express Hsp27 through the HSF1-mediated activation of its transcription [Chen and Currie, 2006] in response to external stress to reduce apoptosis. Several studies have shown that Hsp27 inhibits the apoptosis induced by heat shock [Stege et al., 1995], oxidative stress [Rogalla et al., 1999] and cytotoxicants [Concannon et al., 2003]. Hsp27 expression is increased in several cancers, including those of the prostate [Cornford et al., 2000], breast [Vargas-Roig et al., 1998], kidney [Takashi et al., 1997], and ovary [Langdon et al., 1995], possibly to promote survival of the malignant cells. Such inhibition of apoptosis occurs through the direct binding of Hsp27 to pro-apoptotic proteins and by inhibiting the activation of pro-apoptotic proteins [Pandey et al., 2000]. For example, Hsp27 can bind to procaspase-3 and inhibit its maturation [Concannon et al., 2001]. It can also bind to cytochrome *c* and prevent apoptosome formation. It binds to Bax and prevents its translocation to the mitochondria during apoptosis. It stabilizes the cytoskeleton of cells by binding to actin [Huot et al., 1996], preventing its oligomerization [Hino et al., 2000] in stressed cells. In addition to binding to pro-apoptotic proteins, Hsp27 can enhance cell survival signaling pathways; its overexpression leads to activation of PI3 kinase activity and, consequently, Akt phosphorylation [Wu et al., 2007]. Phosphorylation of Hsp27 is thought to be essential for this function [Mehlen et al., 1995; Benn et al., 2002], but there are reports that suggest that phosphorylation is not essential for this particular function [Martin et al., 1999; Rogalla et al., 1999]. Phosphorylation leads to the breakdown of the oligomeric structure into a predominantly tetrameric structure. It is believed that phosphorylated Hsp27 translocates into the nucleus during the prevention of apoptosis. It has not been fully resolved whether nuclear translocation is an obligatory step to prevent apoptosis. While studies show that nuclear translocation is essential, one study shows it is not [Borrelli et al., 2002]. GSH upregulation was thought to be involved in the protection against apoptosis by Hsp27 [Mehlen et al., 1996], but this may not be universal [Borrelli et al., 2002]. Hsp27 has also

been proposed to inhibit protein synthesis by binding to cap-binding initiation complex (eIF4F) to limit the unfolded protein response in thermally stressed cells [Cuesta et al., 2000].

In the human lens, Hsp27 is present along with other small heat shock proteins, α A- and α B-crystallins. Hsp27 is present predominantly in its phosphorylated form in aged and cataractous lenses [Oya-Ito et al., 2006], including the rat lens (Hsp25) [Chiesa et al., 1997]. The binding of Hsp27 to α B-crystallin makes it more resistant to thermal denaturation [Fu and Liang, 2003]. It freely exchanges subunits with α -crystallin [Bova et al., 2000]. One study showed that Hsp27 upregulation is a possible protective mechanism against interferon- γ -induced apoptosis in human lens epithelial cells [Awasthi and Wagner, 2005]. Further work is needed to understand the precise role of Hsp27 in the human lens. However, in the mouse lens, Hsp25 in its phosphorylated form appears to be important for epithelial cell differentiation into fiber cells [Chiesa et al., 1997].

In general small heat shock proteins contain either a very few or no cysteine residues. This is thought to give them the flexible conformation to fold and refold, which are required during their chaperone function [Fu et al., 2003]. Human Hsp27 has a single cysteine residue in the α -crystallin domain at position 137. This cysteine can undergo oxidation and form a disulfide link with the C137 of another Hsp27. In addition, during cardiac hypoxia-reperfusion injury, Hsp27 is S-thiolated [Eaton et al., 2002a]. A more recent study showed that S-thiolation of the C137 residue resulted in the disaggregation of Hsp27 [Eaton et al., 2002b]. It is unknown whether the disaggregation of Hsp27 results in an alteration of chaperone function, although phosphorylation, which also results in disaggregation, leads to an alteration in the chaperone function (see above). In the murine Hsp25, the single cysteine at position 141 forms an intermolecular disulfide bond in cells, but disulfide formation does not change its chaperone function [Zavialov et al., 1998]. These observations suggest that the single cysteine residue in human Hsp27 could play a role in the protein oligomerization, chaperone, and anti-apoptotic functions.

Hsp27 is ubiquitously present and it is believed to protect cell and tissue injury under many conditions of stress by preventing apoptosis of cells. Under such conditions, Hsp27 is likely to be oxidized. Therefore, it is essential to understand the role of the cysteine in structural alteration, changes in its oligomeric size and alterations in the chaperone activity and anti-apoptotic functions. In this study we report the effects of chemical modification and site-directed mutagenesis of cysteine residue on its structure and functions.

MATERIALS AND METHODS

MATERIALS

Citrate synthase (CS), insulin, 2-p-toluidinyl-naphthalene-6-sulfonate (TNS) and carbonic anhydrase (CA) were from Sigma Chemical Co. (St. Louis, MO). Tris (2-carboxyethyl) phosphine (TCEP)-gel and mammalian protein extraction reagent (MPER) were from Thermo Fisher Scientific (Waltham, MA). All other chemicals used were of analytical grade.

CLONING AND EXPRESSION OF WILD-TYPE (Hsp27_{WT}) AND MUTANT (Hsp27_{C137A}) HUMAN Hsp27

Hsp27_{WT} and Hsp27_{C137A} were cloned with hexa-histidine tag at the N-terminus for bacterial expression and purification. Hsp27_{WT} with an N-terminal hexa-His tag was generated by PCR using the forward primer 5'-CATGCCATGGGGCACCACCACCACCACATGACCG-AGCGCCGCGTCCCCTTCTCG-3' and the reverse primer 5'-CCC-AAGCTTTTACTTGGCGGCAGTCTCATCGGATTT-3'. Hsp27 cDNA (Thermo Scientific Open Biosystems, Huntsville, AL) was used as a template. The amplified PCR product was inserted into the pET 23d vector using *Nco*I and *Hind*III restriction sites. The Hsp27_{C137A} mutant was generated by site-directed mutagenesis using the forward primer 5'-ATTTCGCGGGCCTTCACGCGG-3' and the reverse primer 5'-CCGCGTGAAGGCCCCGGGAGAT-3'. Hsp27 inserted into the pET23d vector was used as a template. For CHO cell expression studies, wild-type Hsp27 (Hsp27_{WT}) was inserted into the pcDNA 3.1(-) mammalian expression vector. Hsp27_{WT} was amplified from Hsp27 cDNA using the forward primer 5'-CTGCAGAATCCATGA-CCGAGCGCCGCGTCCCCTTCTCG-3' and the reverse primer 5'-CCCAAGCTTTTACTTGGCGGCAGTCTCATCGGATTTTGC-3'. *Eco*RI and *Hind*III restriction sites were used for cloning. The Hsp27_{C137A} mutant was generated by site-directed mutagenesis using the primers described above. Hsp27 inserted into pcDNA3.1(-) was used as a template. Plasmids containing either Hsp27_{WT} or Hsp27_{C137A} cDNA were transformed into BL21 (DE3) pLysS *Escherichia coli* for protein purification. One liter of LB medium containing 100 µg/ml ampicillin was inoculated with 50 ml of an overnight culture of BL21 *E. coli* harboring either Hsp27_{WT} or Hsp27_{C137A}, and cultured at 37°C with shaking at 250 rpm until the optical density at 600 nm reached 0.5–0.7. Protein expression was induced using 1 mM IPTG at 37°C for 5 h. Cells were harvested at 6,000g for 15 min and the cell pellet was re-suspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8). Cells were lysed by sonication at 30% amplitude using a Branson digital sonifier (Danbury, CT). Cell lysates were incubated with Ni-NTA resin for 1 h at room temperature according to the manufacturer's instructions (Qiagen, Valencia, CA). The Ni-NTA resin was washed with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8) and the protein was eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8). The protein fractions were pooled and dialyzed against Chelex-100-treated PBS at 4°C. The pooled protein fractions were concentrated using Amicon Ultra-15 centrifugal filters (Millipore). The protein samples were separated by SDS-PAGE, transferred to nitrocellulose membrane by Western blotting and detected by an anti-Hsp27 monoclonal antibody.

CHAPERONE ASSAYS

The chaperone function of Hsp27 was evaluated using two client proteins. The first protein was CS and the assay procedure was performed as described by Biswas et al. [2007]. Briefly, CS (15 µg) was incubated at 43°C in 200 µl of 40 mM HEPES buffer, pH 7.4 in the presence or absence of Hsp27 (0.75 µg) and the light scattering caused by protein aggregation was monitored at 360 nm for 60 min. The Hsp27/CS ratio was 1:20. The second

protein was insulin. The insulin aggregation assay was performed as described by Biswas et al. [2007]. Briefly, 80 μg of insulin in 0.1 M sodium phosphate buffer, pH 7.0 in the presence of 40 mM EDTA and 50 mM DTT was incubated with 13.5 μg of Hsp27. The insulin/Hsp27 ratio was 5.8:1. Light scattering due to protein aggregation was monitored at 360 nm for 60 min.

IN SITU GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH) AGGREGATION ASSAY

Cell lysates were prepared from 10×10^6 cells using MPER and were incubated at 55°C for 3 h to induce GAPDH aggregation. Soluble and insoluble fractions were separated as described by Hayes et al. [2008] and 10 μg of protein was separated by SDS-PAGE. A Western blot was performed and GAPDH was detected using the anti-GAPDH monoclonal antibody (Millipore). Purified GAPDH from rabbit muscle (Sigma Chemical Co.) was used as the positive control.

SURFACE HYDROPHOBICITY AND TRYPTOPHAN FLUORESCENCE

The surface hydrophobicity of Hsp27 was measured using TNS. Hsp27 (100 μg) was incubated with 160 μM TNS (26 mM stock solution prepared in DMSO) in 50 mM sodium phosphate buffer, pH 7.4 at 25°C for 2 h. The fluorescence emission spectrum (350–530 nm) was recorded in a Fluoromax-4 Horiba Jobin Yvon Spectrofluorometer (excitation = 320 nm) with the excitation and emission band passes set at 5 nm. Data were collected at a 0.5 nm resolution. The tryptophan fluorescence of 20 μg of protein in 1.0 ml of water was recorded at an excitation wavelength of 295 nm and an emission wavelength that ranged from 310 to 400 nm with a 5-nm band pass.

FAR UV-CD SPECTROSCOPY

Hsp27_{WT} and Hsp27_{C137A} were each diluted to 0.2 mg/ml in 10 mM sodium phosphate buffer, pH 7.2. Spectra were scanned from 195 to 250 nm. The secondary structure content of the protein was calculated using K₂d software.

ESTIMATION OF BINDING PARAMETERS

This was essentially done as described by Biswas et al. [2007]. Briefly, 12.5 μM Hsp27_{WT} or mutant Hsp27_{C137A} was incubated with 0–15 μM CA for 1 h at 57°C in 50 mM phosphate buffer, pH 7.2 containing 100 mM NaCl. The temperature of the incubations was lowered to 25°C for 1 h. The incubations were then filtered using a 100 kDa centrifugal filter (4,000g) to separate unbound CA from the Hsp27-CA complex. The amount of CA bound by Hsp27 was calculated by subtracting free substrate from the total substrate concentration. The unbound substrate concentration was determined by measuring the absorbance at 280 nm and using an extinction coefficient of $1.9 (\text{mg/ml protein})^{-1} \text{ cm}^{-1}$ for CA. The number of binding sites (n) and dissociation constant (K_d) were determined as previously described [Biswas and Das, 2004].

SUBUNIT EXCHANGE RATE

Fluorescence labeling of Hsp27_{WT} and mutant Hsp27_{C137A} was done using Alexa 350 and Alexa 488 fluorophores (both from Molecular Probes, Invitrogen, Carlsbad, CA) as described by the supplier. Hsp27 (~5 mg each) was dialyzed overnight against 0.1 M sodium bicarbonate, pH 8.3 and then incubated with the fluorescent tag for 1 h at RT. The incubations were subjected to gel filtration on a BioGel P-10 column using PBS as the eluent. The eluate was monitored for protein at the absorbance of 280 nm. The protein fractions were pooled and dialyzed against 4 L of PBS for 36 h. The amount of protein label was determined using formulae:

$$\begin{aligned}\text{For Alexa 350: } & \frac{A_{346} \times \text{dilution factor}}{19,000 \times \text{protein(M)}} \\ \text{For Alexa 488: } & \frac{A_{494} \times \text{dilution factor}}{71,000 \times \text{protein(M)}}\end{aligned}$$

Subunit exchange was accomplished by mixing equal volumes of 0.4 mg/ml Hsp27 in PBS at 37°C. At various time intervals, 5 µl of the reaction mixture was removed and diluted 40-fold in the same buffer. The fluorescence spectra of the samples were recorded from 400 to 600 nm at 37°C in a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ) at an excitation wavelength of 335 nm. The excitation and emission monochromators had a slit width of 5 nm. We measured the intensity at 440 nm and calculated the subunit exchange rate using the following equation: $F(t)/F(0) = D_1 + D_2e^{-kt}$, where $F(t)$ is the fluorescence intensity at 415 nm at various time intervals, $F(0)$ is the fluorescence intensity at 440 nm at $t = 0$, and k is the subunit exchange rate constant. The constants D_1 and D_2 were determined using conditions at which $D_1 + D_2 = 1$ at $t = 0$ and D_1 is the fluorescence intensity at $t = \infty$.

MULTI-ANGLE LIGHT SCATTERING

The molecular size of Hsp27_{WT} and mutant Hsp27_{C137A} was determined by dynamic light scattering measurements. Briefly, the proteins were incubated at 37°C for 1 h and then chromatographed on a TSK G5000PWXL column (Tosoh Bioscience, Inc., San Francisco, CA) using 50 mM phosphate buffer, pH 7.2 containing 0.3 M NaCl. The size-exclusion column was connected to a high performance liquid chromatograph (HPLC) equipped with a refractive index detector (Shimadzu, Columbia, MD) and coupled to multiangle light scattering and quasi-elastic light scattering detectors. The molar mass (M_w) and hydrodynamic radius (R_h) of the proteins were determined with ASTRA (5.1.5) software (Wyatt Technology Corp., Santa Barbara, CA).

S-CARBOXYMETHYLATION OF THE CYSTEINE IN Hsp27

Hsp27_{WT} (1.1 mg) was taken diluted in 1.0 ml of 0.1 M Tris-HCl, pH 8.5. DTT was added to a final concentration of 10 mM and the protein was incubated for 1 h at 37°C. Iodoacetic acid was added to a final concentration of 25 mM and the reactions were incubated for 30 min at RT. DTT was then added to a final concentration of 20 mM to quench the excess iodoacetic acid. The protein was dialyzed against PBS for 18 h.

S-THIOLATION OF THE CYSTEINE IN Hsp27

Hsp27_{WT} or the Hsp27_{C137A} mutant (300 µg each) was diluted in 100 µl of 0.1 M sodium phosphate buffer, pH 7.4 containing 5 mM EDTA. To this mixture, 150 µl of TCEP gel slurry (Thermo Fisher Scientific) was added. Prior to this, the gel was washed three times by centrifugation (1,000g, 2 min each) with 0.1 M sodium phosphate buffer, pH 7.4 with 5 mM EDTA. The protein + gel sample was incubated for 1 h at RT with shaking, followed by centrifugation at 4,000g for 5 min. One hundred microliters of freshly prepared GSSG was added to the supernatant to give a final concentration of 25 mM and the mixture was incubated for 2 h at RT. The protein was then dialyzed against PBS for 18 h. Protein content in the dialyzed samples was estimated by the BCA method.

OVEREXPRESSION OF Hsp27 IN CHO AND HeLa CELLS

Chinese hamster ovary (CHO) cells and HeLa cells (a human cervical cancer cell line) were used for transient transfections of Hsp27_{WT} and the Hsp27_{C137A} mutant. Cells were cultured in HAM's F-12 media supplemented with 10% fetal bovine serum (FBS). Superfect transfection reagent (Qiagen) was used for transfecting the empty pcDNA3.1(-) vector, Hsp27_{WT}, or the Hsp27_{C137A} mutant. Transfections were done for 48 h. Cells were harvested and protein extracts were made using MPER reagent according to the manufacturer's protocol. Protein samples were prepared in Laemmli buffer with or without β-mercaptoethanol. Samples containing 20 µg of protein were separated by SDS-PAGE and subjected to Western blotting. Hsp27 was detected using an anti-Hsp27 (1:1,000 dilution) mouse monoclonal antibody (Cell Signaling, Danvers, MA); an anti-GAPDH (1:10,000 dilution) mouse monoclonal antibody (Chemicon International, Temecula, CA) was used to detect GAPDH, which served as a loading control.

INDUCTION OF APOPTOSIS

Apoptosis was induced after 48 h of transient transfection using 100 nM staurosporine or 20 µM etoposide for 30 h. Cells maintained in culture media containing 0.01% DMSO were used as a control. Apoptosis was also induced by incubating the cells with 1 mM H₂O₂ in media containing 1% fetal bovine serum for 24 h. Cells were stained with Hoechst stain to measure the percentage of apoptotic cells. A total of 300 cells were counted.

CASPASE-3 AND CASPASE-9 ACTIVITIES

Cell lysates were made following apoptosis induction as described above. Caspase-3 activity was detected using 50 µg of protein in 20 mM PIPES, pH 7.2, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose buffer. DEVD-AFC (20 µM) was used as the substrate. Caspase-9 activity was detected using 50 µg of protein in 100 mM MES, pH 6.5, 10% PEG, 0.1% CHAPS, 10 mM DTT buffer. LEHD-AFC (100 µM) was used as the substrate. Samples were incubated for 2 h at 37°C and read at 400/505 nm excitation/emission wavelengths using a Spectramax Gemini XPS spectrofluorometer (Molecular Devices, Sunnyvale, CA).

STATISTICAL ANALYSIS

Data are expressed as the mean $\pm$ SD. Statistical analysis was performed with Statview software. Paired *t*-test was used to determine significant differences between the means, *P* 0.05 was considered significant.

RESULTS

PURIFICATION OF PROTEINS AND MOLECULAR MASS DETERMINATION

His-tagged Hsp27_{WT} and Hsp27_{C137A} mutant were expressed in *E. coli* and purified using a Ni-Agarose column. The protein preparation was found to be pure by SDS-PAGE and western blotting (Fig. 1A, B). We noted that purified proteins underwent fragmentation upon storage at 4°C. Dialysis against Chelex-100-treated PBS before storage prevented such fragmentation. SDS-PAGE and Western blotting of proteins in the absence of the reducing agent showed that most Hsp27_{WT} was present as disulfide bond-linked dimer, while the Hsp27_{C137A} mutant was present as a monomer (Fig. 1C, D). Multi-angle light scattering experiments showed that the molecular mass and hydrodynamic radius were similar for the two proteins (Table I). The hydrodynamic radii of the two protein preparations were similar, suggesting that they were made up of the same number of subunits, that is, ~33 subunits of 27 kDa each. Addition of 5 mM DTT caused a slight reduction in these parameters in both Hsp27_{WT} and Hsp27_{C137A}. Thus, the effect seen with DTT was not due to reduction of the disulfide bond in Hsp27_{WT}. These observations imply that disulfide formation does not influence protein oligomerization in Hsp27.

STRUCTURAL CHARACTERISTICS

Intrinsic tryptophan fluorescence was used to assess changes in protein tertiary structure. The mutant protein had tryptophan fluorescence identical to the wild-type protein (Fig. 2A) suggesting that the C137A mutation did not cause structural changes in the protein. The far UV-CD spectra showed no apparent differences; the α -helix, β -sheet and random coil contents were similar (Table II).

The chaperone function in small heat shock proteins is attributed to their hydrophobicity [Nakamoto and Vigh, 2007], although a direct quantitative relationship between the chaperone function and hydrophobicity may not exist [Reddy et al., 2006]. Nevertheless, it is generally believed that hydrophobic patches in these proteins bind to client proteins and inhibit their aggregation. Therefore, we determined if surface hydrophobicity was changed in the mutant protein. As shown in Figure 2B the surface hydrophobicity, determined by TNS binding, was similar in Hsp27_{WT} and the Hsp27_{C137A} mutant.

CHAPERONE FUNCTION AND BINDING CONSTANT

The chaperone function of Hsp27_{WT} and the Hsp27_{C137A} mutant proteins was tested using CS and insulin as client proteins. The former was used in a thermal aggregation assay and the latter in a chemical-induced aggregation assay. At a 1:20 ratio of Hsp27: CS, there was a ~30% and ~35% decrease in CS aggregation with Hsp27_{WT} and Hsp27_{C137A} protein, respectively (Fig. 3A, B). With insulin as the client protein and a ratio of 1:5.8 of Hsp27/Insulin, there was 65% and 70% inhibition with Hsp27_{WT} and Hsp27_{C137A}, respectively

(Fig. 3C, D). In both cases, the difference was statistically insignificant. We would like to point out that in the insulin aggregation assay, we used DTT to induce protein aggregation, which could have reduced disulfide bond in Hsp27_{WT}. Next we checked if the in situ chaperone function of Hsp27 is altered by the C137A mutation. For this we transiently overexpressed Hsp27_{WT} and the Hsp27_{C137A} mutant protein in CHO cells. The expression levels were similar for the two proteins (Fig. 4A). GAPDH aggregation was used as the readout in this experiment. In all cell lysates incubated at room temperature, GAPDH remained in the soluble fraction. When cell lysates from empty vector-transfected cells were incubated at 55°C, GAPDH was found exclusively in the insoluble pellet fraction, suggesting total aggregation of all soluble GAPDH (Fig. 4B). However, in cell lysates transfected with either Hsp27_{WT} or Hsp27_{C137A}, nearly 50% of GAPDH was present in the soluble fraction, suggesting that both proteins inhibited thermal aggregation of GAPDH to an equal extent. The binding constant determination showed that replacement of the cysteine residue with alanine did not significantly alter either the number of CA molecules that can bind Hsp27 during chaperoning or the binding affinity (K_d) of Hsp27 for CA (Table III).

EFFECT OF CARBOXYMETHYLATION AND S-THIOLATION ON THE CHAPERONE FUNCTION

To further investigate the importance of C137 in the chaperone function, we carboxymethylated the cysteine residue in the Hsp27_{WT} protein and checked the chaperone function. As seen in Figure 5A, B, carboxymethylation had no effect on the chaperone function using either CS or insulin as client proteins. The carboxymethylated protein showed a monomeric band at 27 kDa in SDS-PAGE under non-reducing conditions, unlike the unmodified protein that showed the dimeric form at 54 kDa (Fig. 5C). Carboxymethylation did not change the monomeric nature of the mutant protein. Thus, it appears that regardless of whether the protein exists as a disulfide-linked dimer or a monomer in its native state, its chaperone function remains the same.

We then performed S-thiolation of C137 using TCEP gel and GSSG. S-thiolation resulted in no significant change in the chaperone function using CS as the client protein (Fig. 5D), again affirming the notion that C137 in either the oxidized or reduced form does not affect the chaperone function of Hsp27.

SUBUNIT EXCHANGE RATE

Next, we determined if the subunit exchange rate is affected by the mutation. To study this subunit exchange rate, we labeled Hsp27_{WT} and Hsp27_{C137A} proteins with either Alexa 350 or Alexa 488. While the subunit exchange rate for the homopolymer of Hsp27_{WT} is somewhat similar to that of Hsp27_{WT}-Hsp27_{C137A}, it was nearly threefold higher for the homopolymer of Hsp27_{C137A} relative to the homopolymer of Hsp27_{WT} protein (Fig. 6).

ANTI-APOPTOTIC FUNCTION

The effect of the C137A mutation on the anti-apoptotic function of the protein was investigated in CHO cells transiently transfected with either Hsp27_{WT} or Hsp27_{C137A}. The protein expression levels of Hsp27_{WT} and Hsp27_{C137A} were similar (Fig. 4A). We used staurosporine and etoposide for the induction of apoptosis. Transfection of the empty vector

served as the control. Treatment of cells transfected with the empty vector with 100 nM staurosporine for 30 h resulted in nearly 65% of the cells being apoptotic (Fig. 7A). Transfection with Hsp27_{WT} significantly inhibited apoptosis ($P < 0.0001$) by 40%. Transfection with Hsp27_{C137A} showed slight but statistically insignificant decrease in the inhibition of apoptosis (~37%). In empty vector-transfected cells, 20 μ M etoposide brought about apoptosis in 62% of those cells after 30 h of treatment (Fig. 7B). Transfection with either Hsp27_{WT} or Hsp27_{C137A} significantly inhibited apoptosis ($P < 0.0001$) by about 30% in each case.

EFFECT ON CASPASE ACTIVITIES

To determine if the inhibition of apoptosis occurred as a result of lower caspase activities, we measured the activities of caspase-3 and -9 in 100 nM staurosporine-treated (30 h) cells transfected with Hsp27. Both Hsp27_{WT} and Hsp27_{C137A} reduced the caspase-3 activity to a similar extent (Fig. 7C). Similarly, caspase-9 activity was reduced by the two proteins (Fig. 7D). Together these data suggest that neither C137 nor Hsp27 dimerization by disulfide bond formation is necessary for Hsp27 to function as an anti-apoptotic protein against staurosporine and etoposide.

ANTI-APOPTOTIC FUNCTION AGAINST OXIDATIVE STRESS

CHO cells were incubated with 1 mM H₂O₂ for 24 h. During this incubation, the FBS concentration was reduced from 10% to 1% to limit the direct interaction of H₂O₂ with the FBS components. SDS-PAGE of the cell lysates in the absence of β -mercaptoethanol, followed by Western blotting using a Hsp27 monoclonal antibody showed that H₂O₂ treatment induced disulfide-linked dimers of Hsp27 in cells transfected with Hsp27_{WT} (Fig. 8A). Such dimer formation was not observed in cells transfected with Hsp27_{C137A}. These results are in agreement with a previous report [Zavialov et al., 1998] and suggest that oxidative stress causes the dimerization of Hsp27 in cells. We then investigated whether C137 is required for the inhibition of apoptosis induced by 1 mM H₂O₂ in CHO cells. CHO cells were transfected with plasmids containing Hsp27_{WT} and Hsp27_{C137A}. The protein expression levels were similar in both cases (Fig. 8B). H₂O₂ caused apoptosis in nearly 80% of empty vector-transfected cells (Fig. 8C). However, this number was reduced to 53% in cell transfected with Hsp27_{WT}. The apoptotic cell number was significantly higher in cells transfected with the Hsp27_{C137A} mutant (60%) relative to cells transfected with Hsp27_{WT}. To determine if similar effects are seen in human cells, we transiently transfected HeLa cells with Hsp27_{WT} and Hsp27_{C137A} and treated with 1 mM H₂O₂ for 24 h. Similar to CHO cells, the apoptotic cell number was significantly higher in HeLa cells transfected with the Hsp27_{C137A} mutant (67%) relative to cells transfected with Hsp27_{WT} (55%) (Fig. 8D). However, in both CHO and HeLa cells, the mutant protein still showed significant protection against 1 mM H₂O₂-induced apoptosis ($P < 0.0001$). This was further confirmed by caspase-3 and -9 activities in CHO cells, which were 17% and 11% higher in Hsp27_{C137A} cells than in Hsp27_{WT} cells, respectively (Fig. 8E, F). Together these results suggest that C137, possibly through dimerization, is only partly essential for the inhibition of apoptosis under oxidative stress.

DISCUSSION

The purpose of this study was to determine the role of the cysteine residue in (1) structural characteristics, (2) protein oligomerization, (3) chaperone activity and (4) anti-apoptotic function of human Hsp27. We employed chemical modification and site-directed mutagenesis of the cysteine residue in our study. SDS-PAGE results showed that the wild-type protein is disulfide linked, unlike the mutant protein, which was not. The disulfide bond in the wild-type protein, its reduction by DTT or replacement of the cysteine residue to alanine had negligible effects on either the secondary structure or oligomeric size of the protein. Our data are somewhat similar to the observation that oxidation of the lone cysteine (C141) in Hsp25 did not change its secondary structure [Zavialov et al., 1998] and mutation of two cysteine residues in human α -crystallin had little effect on the secondary structure [Chen et al., 2001]. Thus, it is likely that cysteine residues in small heat shock proteins may not contribute to structural characteristics.

Our results also suggest that the replacement of C137 with alanine does not alter the chaperone function and are in agreement with previous studies that showed no change in the chaperone function upon mutation of the cysteine in Hsp27 [McHaourab et al., 1997] or oxidation of the lone cysteine (C141) in Hsp25 [Zavialov et al., 1998]. However, the present results differ from those from a previous study, which revealed that cysteine mutation in the related α A-crystallin, the replacement of C131 or C142 with an isoleucine, brought about a profound reduction in the chaperone function of the protein [Kanade et al., 2009]. This suggests that Hsp27 does not interact with its client proteins at its cysteine residue unlike α A-crystallin, which is likely to interact with client proteins at its two cysteine residues.

S-Thiolation of Hsp27 has been shown to occur during cardiac ischemic injury [Eaton et al., 2002b]. S-Thiolation of Hsp27 results in the disaggregation of oligomers, as is seen after phosphorylation. While oligomer disaggregation by phosphorylation increases the chaperone function [Hayes et al., 2009], disaggregation by S-thiolation (if it occurred in our study) does not change the chaperone function. This suggests that even though both post-translational modifications induce disaggregation, the outcome on the chaperone function is different.

The subunit exchange rate was higher in Hsp27_{C137A} than Hsp27_{WT}. This could be due to the monomeric nature of subunits in Hsp27_{C137A} unlike the dimeric disulfide-linked subunits of Hsp27_{WT}. Similar lower subunit exchange rate was observed in human α A-crystallin in which cysteine residues were oxidized [Liang and Fu, 2002]. The subunit exchange rate of Hsp27 is at least twofold higher than α -crystallin [Bova et al., 2000; Biswas et al., 2008]. The higher subunit exchange rate seen in the mutant protein did not translate to increased chaperone function, contrary to the observations with the related α A-crystallin [Ghahghaei et al., 2007; Biswas et al., 2008]. The results in the present study are in agreement with a previous report that the subunit exchange rate in α -crystallin is unrelated to the chaperone function [Aquilina et al., 2005]. The C-terminus of small heat shock proteins appears to be important for subunit exchange [Aquilina et al., 2005]. It is not clear how the mutation of C137, which is in the “ α -crystallin” core domain and has no effect on the protein structure, but enhances the subunit exchange rate.

Our study showed that the cysteine residue does not take part in the anti-apoptotic function of Hsp27 against chemical induced apoptosis. Our results are different from those obtained in previous studies, which showed a loss of the anti-apoptotic function of Hsp27_{C137A} against etoposide [Bruey et al., 2000] and murine Hsp25_{C141A} against staurosporine [Diaz-Latoud et al., 2005]. In both cases the single cysteine residue was replaced with alanine. It is not apparent why the two studies on Hsp27 showed contrasting results. It is possible that the difference is due to different cell types studied. While our study used CHO cells, Bruey et al. used a human leukemic monocyte lymphoma cell line.

Inhibition of oxidative stress-induced apoptosis by Hsp27 is likely to occur through the upregulation of enzymes that increase intracellular GSH [Arrigo, 2001]. We can only speculate as to why the Hsp27_{C137A} mutant is less effective than Hsp27_{WT} in preventing H₂O₂-induced apoptosis. One possibility is that by allowing its cysteine to undergo oxidation, Hsp27 might protect other cell components from oxidative damage and inhibit apoptosis. Such a phenomenon cannot occur with the cysteine-lacking mutant. It may also be possible that the mutant protein, unlike the WT protein, is incapable of upregulating enzymes that elevate intracellular GSH. Another possibility is that H₂O₂ induced oxidation of other amino acids (tryptophan and methionine) in Hsp27_{C137A} that made it a weaker anti-apoptotic protein. Thus, the role of C137 in the prevention of H₂O₂-induced apoptosis appears to be minimal at best.

In summary, we have demonstrated that even though the lone cysteine in human Hsp27 can undergo oxidation to form disulfide-linked dimers of the protein, it plays no significant role in either the chaperone or anti-apoptotic functions of the protein. However, we cannot rule out role of the lone cysteine in other cellular functions of Hsp27, such as binding to actin in stressed cells.

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Abbreviations used

Hsp27	heat shock protein 27
Hsp27_{WT}	human heat shock protein 27 wild-type
Hsp27_{C137A}	human heat shock protein 27 cysteine-alanine mutant
CS	citrate synthase
Hsp25	heat shock protein 25
CA	carbonic anhydrase
TCEP	Tris (2-carboxyethyl) phosphine

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSH	reduced glutathione
GSSG	oxidized glutathione
DTT	dithiothreitol
MAPKAP	mitogen-activated protein kinase-activated protein
RT	room temperature
TNS	2-p-toluidinyl-naphthalene-6-sulfonate
MPER	mammalian protein extraction reagent
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)

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Fig. 1. Disulfide bond formation in Hsp27. SDS-PAGE (A) and Western blotting (B) of purified Hsp27_{WT} and Hsp27_{C137A} proteins. Lane 1, molecular weight markers; lane 2, Hsp27_{WT}; lane 3, Hsp27_{C137A}. SDS-PAGE (C) and Western blotting (D) in the presence (lanes 3 and 5) and absence of β-mercaptoethanol (lanes 2 and 4). Lane 1, molecular weight markers; lanes 2 and 3, Hsp27_{WT} in the presence and absence of β-mercaptoethanol; lanes 4 and 5, Hsp27_{C137A} in the presence and absence of β-mercaptoethanol. Arrows indicate Hsp27 bands. Arrows indicate dimer or monomer Hsp27.

Fig. 2. Effect of the C137A mutation on the structure and surface hydrophobicity of Hsp27. The tryptophan fluorescence (A) and surface hydrophobicity, which was assessed by TNS fluorescence (B), were similar between the Hsp27_{WT} and Hsp27_{C137A} proteins.

Fig. 3. Role of C137 in the chaperone function of Hsp27. A, B: Chaperone assay using CS as the client protein; (C, D) chaperone assay using insulin as the client protein. Bar graphs (B, D) are the mean $\pm$ SD from three independent experiments. $*P = < 0.05$. Blank = buffer alone.

Fig. 4.

In situ chaperone function of Hsp27 in CHO cells. CHO cells were transiently transfected with plasmids carrying Hsp27_{WT} and Hsp27_{C137A}. Cellular expression of Hsp27 was similar between the two transfections (A). Cell lysates were incubated at 55°C to induce precipitation and insolubilization of GAPDH. The lysate was separated into supernatant (S) and pellet (P) by centrifugation and the two fractions were analyzed by Western blotting using a monoclonal antibody against GAPDH (B). EV, empty vector.

Fig. 5.

Carboxymethylation of cysteine has no effect on the chaperone function of Hsp27. The chaperone function was assessed using CS (A) and insulin (B) as the client proteins. The unmodified (UM) and carboxymethylated (CM) proteins showed similar chaperone functions. SDS-PAGE of Hsp27_{WT} and Hsp27_{C137A} in the presence or absence of β -mercaptoethanol is shown in (C). Hsp27_{C137A} was also carboxymethylated along with Hsp27_{WT} for comparison. Lane 1, molecular weight markers; lane 4, unmodified Hsp27_{WT}; lane 5, carboxymethylated Hsp27_{WT}. Carboxymethylated Hsp27_{WT} did not form the dimer, unlike unmodified Hsp27. Hsp27_{C137A} was subjected to similar treatment and is shown in lanes 2 and 3. Similarly, S-thiolation (M) of the cysteine residue with GSSG did not affect the chaperone function of Hsp27 (D). The chaperone function was assessed using CS as the client protein. Arrows indicate dimer or monomer of Hsp27. Blank = buffer alone.

Fig. 6.

Effect of the C137A mutation on the subunit exchange rate of Hsp27. Subunit exchange within the Hsp27_{WT} and Hsp27_{C137A} homopolymers, and between Hsp27_{WT} and Hsp27_{C137A} (Alexa 388 or 450-labeled, respectively) was measured at 37°C. An equal amount (0.4 mg/ml) of each labeled protein was mixed together at 37°C and the decrease in relative fluorescence intensity at 440 nm was determined. The subunit exchange rate was calculated using the equation $F(t)/F(0) = D_1 + D_2e^{-kt}$.

Fig. 7.

Role of C137A in the anti-apoptotic function of Hsp27. Hsp27_{WT} and Hsp27_{C137A} were transiently expressed in CHO cells. Apoptosis was induced by treating cells with 100 nM staurosporine for 30 h (A) or 20 μ M etoposide for 30 h (B). Apoptosis was assessed by Hoechst staining. Capase-3 (C) and caspase-9 (D) activities in staurosporine-treated cells were measured using fluorescent peptide substrates. $P < 0.0001$. In each case $n = 3$.

Fig. 8.

Oxidative stress causes intermolecular disulfide bond formation in Hsp27. CHO cells were transiently transfected with vector carrying either Hsp27_{WT} or Hsp27_{C137A}. Cells were then incubated with 1 mM H₂O₂ for 24 h. Cell lysates were analyzed by SDS–PAGE in the absence of β-mercaptoethanol. Hsp27_{WT} formed a dimer upon H₂O₂ treatment, while Hsp27_{C137A} did not (A). Protein expression levels of Hsp27_{WT} and Hsp27_{C137A} were similar in transfected cells (B). Apoptosis in CHO cells (C) and HeLa cells (D) transfected with Hsp27_{WT} or Hsp27_{C137A} and treated with 1 mM H₂O₂ for 24 h. Caspase-3 (E) and caspase-9 (F) activities in CHO cells transiently transfected with vector carrying either Hsp27_{WT} or Hsp27_{C137A} and incubated with H₂O₂ as above. **P* < 0.0001 and ***P* = 0.05. EV, empty vector. In each case n = 3.

TABLE I

Molecular Mass and Hydrodynamic Radius of Purified Hsp27

Protein	Molecular mass (g/mol)	Hydrodynamic radius (nm)
Hsp27 WT	1.080E6	9.13
Hsp27 WT—DTT treated	0.972E6	8.70
Hps27 Mt	1.049E6	9.91
Hsp27 Mt—DTT treated	0.978E6	9.31

TABLE II

Secondary Structure Characteristics of Purified Hsp27

Protein	Alpha-helix (%)	Beta-sheet (%)	Random coil (%)
Hsp27 _{WT}	8	44	48
Hsp27 _{C137A}	9	43	48

Spectra from protein samples (0.2 mg/ml in 10 mM phosphate buffer, pH 7.2) were recorded with a 1.0 mm path length cell. Data interval was 0.2 nm. Different secondary structural elements in Hsp27_{WT} and Hsp27_{C137A} were calculated using K2d software.

TABLE III

Determination of Number of Binding Site (n) and Dissociation Constant Values for Interaction of Hsp27 With CA

System studied	n	K _d (mM)
HSP27 _{WT} + CA	1.49	1.87
HSP27 _{C137A} + CA	1.56	1.47