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Conformational Changes in Human Hsp70 Induced by High Hydrostatic Pressure Produce Oligomers with ATPase Activity but without Chaperone Activity

Thaís L. S. Araujo,[†] Julio Cesar Borges,[‡] Carlos H. Ramos,[§] José Roberto Meyer-Fernandes,[†] Reinaldo S. Oliveira Júnior,[∥] Pedro G. Pascutti,[∥] Debora Foguel,*[,†] and Fernando L. Palhano*[,†]

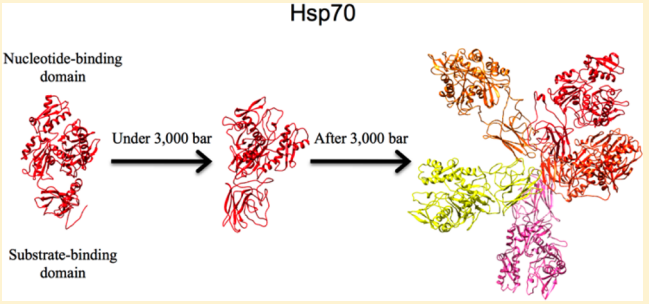
[†]Instituto de Bioquímica Médica Leopoldo de Meis, Programa de Biologia Estrutural, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-590, Brazil

[‡]Instituto de Química de São Carlos, Universidade de São Paulo, USP, São Carlos, SP 13560-970, Brazil

[§]Institute of Chemistry, University of Campinas UNICAMP, Campinas, SP 13083-970, Brazil

[∥]Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-590, Brazil

ABSTRACT: We investigated the folding of the 70 kDa human cytosolic inducible protein (Hsp70) *in vitro* using high hydrostatic pressure as a denaturing agent. We followed the structural changes in Hsp70 induced by high hydrostatic pressure using tryptophan fluorescence, molecular dynamics, circular dichroism, high-performance liquid chromatography gel filtration, dynamic light scattering, ATPase activity, and chaperone activity. Although monomeric, Hsp70 is very sensitive to hydrostatic pressure; after pressure had been removed, the protein did not return to its native sate but instead formed oligomeric species that lost chaperone activity but retained ATPase activity.



Chaperones help proteins reach their native state in the crowded intracellular environment.[1] The 70 kDa heat shock protein (Hsp70) is an important ATP-dependent intracellular chaperone that is conserved among various kingdoms. Hsp70 is critical in cell signaling and is considered an important drug target.[2] Hsp70 has two domains: an N-terminal ATPase domain (NBD, 45 kDa) and a substrate-binding domain (SBD, 25 kDa). The ATPase cycle of Hsp70 NBD induces conformational changes in Hsp70; the ATP-bound state has a low affinity for unfolded protein substrates compared to that of the ADP-bound state.[3] The folding of at least 20% of the newly translated proteins in eukaryotic cells is assisted by Hsc70 (the constitutively expressed form of Hsp70), and most of these are believed to be multidomain proteins.[4] Although protein folding assisted by Hsp70 is well-documented,[1−3] the folding process of Hsp70 itself remains elusive.

Most experiments aiming to understand the folding of Hsp70 have been performed with the *Escherichia coli* Hsp70 homologue, DnaK.[5−10] The unfolding process of DnaK involves two folding intermediates during thermal[10] and acidic denaturation.[9] After thermal denaturation, DnaK recovers its native structure,[6] while bovine brain Hsc70[6] and human Hsp70[11] aggregate irreversibly. The main goal of this study was to investigate the folding process of cytosolic inducible eukaryotic Hsp70 through the use of a gentler perturbing agent, namely high hydrostatic pressure (HHP). HHP in the range of 1−3000 bar has been used as an effective tool to dissociate oligomeric proteins;[12,13] most monomeric proteins are not completely unfolded by HHP treatment in this pressure range, mainly because the protein−protein H-bonds are replaced by protein−water H-bonds upon HHP-induced denaturation, a reaction with no net change in volume.[13] Recently, Roche and colleagues showed that pressure unfolds proteins by eliminating cavities that are present in the folded state but absent in the unfolded state.[14] We observed that human Hsp70 forms oligomers after being unfolded by high hydrostatic pressure. One of the interesting features of Hsp70 is that the oligomers produced by a cycle of pressurization and depressurization can bind ATP but cannot refold an Hsp70 substrate. Our data suggest that during Hsp70 folding, this chaperone must be helped by some other chaperone to reach its final native structure.

EXPERIMENTAL PROCEDURES

Protein Purification. The His-tagged human 70 kDa heat shock cytosolic inducible protein (Hsp701A) was expressed and purified as described previously,[11] with the exception of ethylenediaminetetraacetic acid, which was omitted in our protocol. The protein was submitted to overnight treatment

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with alkaline phosphatase (0.5 unit/mg) to obtain a nucleotide-free preparation.[11,15] The Hsp70 was lyophilized and stored at 4 °C under vacuum. Before each experiment, the Hsp70 powder was resuspended in HKM buffer containing 25 mM HEPES-KOH, 50 mM KCl, and 5 mM $MgCl_2$ (pH 7.6). The protein concentration was determined using an extinction coefficient of 33600 M^{-1} cm^{-1} at 280 nm. Hsp70 was incubated, when indicated, with 2 mM ATP (Sigma), 2 mM ADP (Sigma), and 60 μM NRLLLTG peptide (Genemed Synthesis, Inc.).

Spectroscopic Measurements under Pressure. We used a high-pressure cell equipped with optical windows from ISS Inc. (Champaign, IL) and a circulating bath, which allowed us to control the temperature during compression and decompression (25 °C). Pressure titration experiments were performed by increasing the pressure in intervals of 15 min. This period of time was used because we observed that under 2900 bar, the Trp fluorescence of Hsp70 reached equilibrium after pressurization for 5 min (data not shown). To test the reversibility, we performed two experiments in which we released the pressure completely or in a stepwise manner. During the stepwise release, we allowed 15 min for equilibrium to be reached after each pressure point. In both cases, we observed the oligomerization of Hsp70 but could not obtain the reaction volume for unfolding. The pressure was increased in steps of 138 bar (1−410 and 830−2070 bar) and 210 bar (410−830 and 2070−2900 bar). Fluorescence spectra were recorded on an ISS K2 spectrofluorometer (ISS Inc.) by setting the excitation to 295 nm and collecting the emission in the range of 315−380 nm. The mean energy of the fluorescence emission at pressure p evaluated by the center of spectral mass $\langle \nu_p \rangle$ is given by

$$\langle \nu_p \rangle = \left(\sum \nu_i F_i\right)/\left(\sum F_i\right) \quad (1)$$

where F_i is the fluorescence emitted at wavelength ν_i. The reaction extension (α) is related to $\langle \nu_p \rangle$ by the expression

$$\alpha = (\langle \nu_p \rangle - \langle \nu_i \rangle)/(\langle \nu_i \rangle - \langle \nu_f \rangle) \quad (2)$$

where $\langle \nu_i \rangle$ and $\langle \nu_f \rangle$ are the initial and final values, respectively, of the center of spectral mass in nanometers and $\langle \nu_p \rangle$ is the center of spectral mass at pressure p.

The light scattering (LS) increase was measured to evaluate the aggregation of the protein.

Molecular Dynamics Simulations. We used the only available full-length structure of Hsp70, bovine Hsc70 [Protein Data Bank (PDB) entry 1YUW], as a model.[15] We performed two independent experiments as described below. All-atom molecular dynamics (MD) simulations in explicit water were performed for Hsp70 with GROMACS version 4.0.4 using the GROMOS96 force field (ffG53a6) parameter set.[16−18] The setup for simulation was as follows. As a preliminary step, the protein was placed in a cubic box with at least 12 Å between the edge of the box and the protein surface. The box was filled with water molecules using the spc216 water model, an extended simple point charge (SPC/E) category.[16,17] The Na^+ and Cl^- ions were used to simulate an ionic strength of 100 mM. The charge of the protein was neutralized by addition of an excess of counterions. The ionization of the amino acid residues was set such that all carboxylates, including the C-terminus of SBD_{1-381} and $NBD_{396-554}$, were ionized, and all arginine and lysine side chains were positively charged. MD simulations were conducted under *NPT* conditions with a constant number of moles, pressure, and temperature, using Berendsen's coupling algorithm (p = 1 bar; τ_p = 0.5 ps; T = 310 K; τ_T = 0.1 ps). A 12 Å cutoff was used for the van der Walls interactions. The long-range electrostatic interactions were treated using the Reaction Field algorithm. The energy of each system was minimized using the steepest descent algorithm, followed by a conjugated gradient minimization. MD simulations were performed using the LINCS algorithm to constrain bond lengths, and periodic boundary conditions were applied in all directions. The resulting structures were simulated for 1.0 ns, while the protein's position was restrained, to equilibrate the protein with the solvent. The system was then simulated for 1.0 ns with no constrictions. After this equilibration, a MD run was performed for 30 ns with a 2 fs time step. The initial velocities of all atoms in the systems were randomly generated according to a Maxwell−Boltzmann distribution at 310 K. After these minimization and equilibration steps, each SBD and NBD system was simulated for 100 ns at 3000 bar.

Cavity Volume Calculations. The cavity volumes were calculated with McVol.[19] PDB entry 1YUW was used, and the volumes were calculated for NBD_{1-381} and $SBD_{396-554}$. We observed cavity volumes of 1579 and 358 $Å^3$ for the NDB and SBD, respectively. It is important to note that the PDB entry (1YUW) used is missing the last 87 amino acids in the helical lid subdomain of the SBD. In other words, we might be underestimating the cavities that are present in the SBD. ΔV was calculated with McVol using the average of the volume differences between PDB entry 1YUW and the two PDB entries generated by the MD simulations conducted under 3000 bar, as described in Molecular Dynamics Simulations.

Size-Exclusion Chromatography. High-performance liquid chromatography (HPLC) was conducted in a Superdex 200 10/300 GL instrument (GE Healthcare), at room temperature, using an HPLC system (Shimadzu SPD-10A). The system was equilibrated in buffer HKM, and a flow rate of 0.5 mL/min was used. The sample elution was monitored by the absorbance at 280 nm.

Atomic Force Microscopy (AFM). Samples were placed directly onto freshly cleaved mica for 10 min in a volume of 50 μL. Then, the samples were washed five times with 50 μL of ultrapure water and air-dried overnight. Tapping-mode AFM in air was performed using an Asylum MFP-3D BIO MFA AFM instrument (Asylum Research, Santa Barbara, CA). Rectangular silicon cantilevers with a resonance frequency of 70 kHz and a nominal spring constant of 2 N/m were used. Samples were imaged at scan rates of 2.0 Hz, and 512 × 512 pixels were collected per image. At least three regions of each surface were investigated to confirm the homogeneity of the samples.

Dynamic Light Scattering. The samples before or after pressure titration were filtered (0.22 μm) before being analyzed. Triplicate samples were examined in plastic cuvettes using ZetaSizer nano. The radii of the particles were recorded as a frequency distribution curve, and the average radius was calculated.

Circular Dichroism Measurements. We used 3 μM Hsp70 in HKM buffer containing 25 mM HEPES-KOH, 50 mM KCl, and 5 mM $MgCl_2$ (pH 7.6). Circular dichroism (CD) measurements were performed in a Jasco-715 spectropolarimeter (Jasco Corp., Tokyo, Japan) using a 1.0 mm path-length quartz cuvette, and data were obtained from scans at a speed of 50 nm/min. The baselines (buffer alone) were subtracted from the corresponding spectra.

Trypsin Digestion. Hsp70 (2 μM, 7 μg) after one cycle of pressurization and depressurization was incubated in the presence or absence of 10 mM ATP for 5 min at room temperature and incubated with 0.007 μg of trypsin [trypsin from bovine pancreas (Sigma)] in HKM buffer for 0, 15, and 30 min at 37 °C. The reaction was stopped by the addition of sodium dodecyl sulfate (SDS) sample buffer and boiling for 5 min. The samples were resolved via a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel stained with Coomassie Blue.

ATPase Assay. Hsp70 (1 μM) before or after pressure was incubated in HKM reaction buffer (0.5 mL) containing 250 μM of ATP and [γ-^{32}P]ATP (10^4 Bq/nmol of ATP) for 20 min at room temperature. The reaction was stopped with 1 mL of activated charcoal (20 g of charcoal/100 mL of 0.1 N HCl). The samples were mixed and centrifuged at 1500*g* for 15 min at 4 °C.[20] The supernatant was removed, and the radioactivity was measured in scintillation fluid (2 g of PPO in 1 L of toluene). The ATPase activity was calculated after subtracting the nonspecific ATP hydrolysis activity measured in the absence of Hsp70.

Luciferase Refolding. Firefly luciferase (0.1 μM, Quantilum Recombinant Luciferase, Promega) in the presence of 2.7 μM Hsp70 and 1 μM Hdj1 (human recombinant Hsp40, Assay designs) was denatured for 20 min at 42 °C in HKM buffer supplemented with 5 mM DTT (Vetec) and 2 mM ATP. Hsp70 was used before pressure or after a 2 h return to the atmospheric pressure after one cycle of compression and decompression. Refolding was started when the temperature was decreased to 30 °C and addition of 5% nuclease-treated rabbit reticulocyte lysate (Promega) and 2.5 mM ATP. After luciferase refolding had been conducted for 2 h, a 1 μL aliquot was diluted in 125 μL of HKM buffer supplemented with 2 mM DTT and 2 mM ATP. After addition of 100 mM beetle luciferin potassium salt (Promega), the luminescence was measured (0.1 s) in a plate reader (VICTORT MX4, PerkinElmer). The enzymatic activity of native luciferase incubated with 2.7 μM Hsp70 and 1 μM Hdj1 and processed as described previously, except for the incubation for 20 min at 42 °C, was set to 100%.

Cross-Linked with Glutaraldehyde. Hsp70 (3.2 μM) before or after pressure (50 μL) was incubated with 0.1% glutaraldehyde for 4 min at room temperature. We also performed glutaraldehyde experiments in the absence or presence of 2 mM ATP. The reaction was stopped with 5 μL of 1 M Tris-HCl (pH 8.0), and the samples were boiled in the presence of 2% SDS for 5 min. The samples were resolved on a 7% SDS–PAGE gel stained with Coomassie Blue.

RESULTS AND DISCUSSION

The change in Hsp70 structure during or after high-pressure treatment was monitored by the intrinsic fluorescence of tryptophan (Trp). Hsp70 has two Trp residues, one in each domain. Figure 1 shows the effects of increasing pressure at 25 °C on the structure of Hsp70. Increasing the HHP to 2900 bar led to a red shift in the center of spectral mass of Trp fluorescence [~5 nm (Figure 1)], suggesting the denaturation of Hsp70. This 5 nm shift in the center of spectral mass of Trp induced by HHP treatment was comparable to that induced by the incubation of Hsp70 with 6 M guanidinium hydrochloride for 24 h (Figure 1). However, we cannot exclude the possibility that the HHP treatment induces an interdomain interaction in Hsp70.

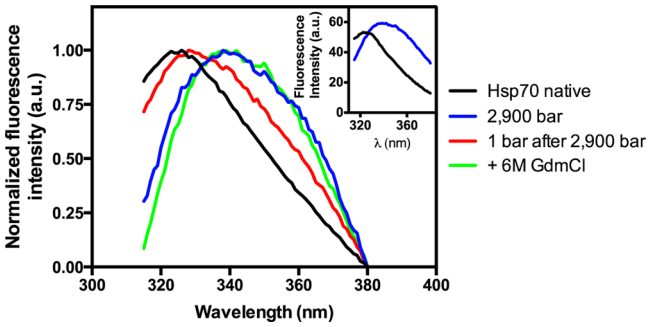

Figure 1. Tryptophan fluorescence spectra of Hsp70. Apo-Hsp70 (3 μM) before pressure (black), under a pressure of 2900 bar (blue), and after pressure titration (red). As a comparison, we incubated Hsp70 in 6 M guanidinium hydrochloride for 24 h (green). The inset shows non-normalized tryptophan fluorescence spectra of Hsp70 before pressure (black) and under a pressure of 2900 bar (blue). Excitation was at 295 nm and emission at 315–380 nm. HKM buffer contained 25 mM HEPES-KOH, 50 mM KCl, and 5 mM $MgCl_2$ (pH 7.6). The temperature was 25 °C.

Next, we investigated whether the presence of nucleotides changes the stability of Hsp70 during pressure treatment. ADP (Figure 2, filled blue circles) and ATP (not shown because of the similarity of its data to the ADP data) promoted an increase in the thermodynamic stability compared to that of the apo-Hsp70 state.

After decompression (Figure 2, empty black circle), the Trp fluorescence did not return to its original value, indicating the irreversibility of the changes in the tertiary structure of Hsp70

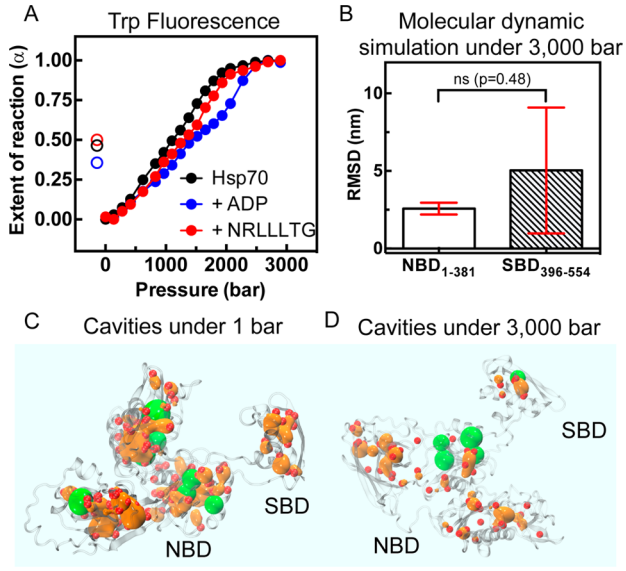

Figure 2. Effect of HHP on the structure of Hsp70. (A) Effect of a nucleotide (ADP) or substrate (peptide NRLLLTG) on the pressure-mediated denaturation of Hsp70. The extent of reaction (α) as a function of pressure was calculated using the center of spectral mass of Trp emission of 3 μM Hsp70. Data for apo-Hsp70 (black) and Hsp70 in the presence of 60 μM peptide NRLLLTG (red) or 2 mM ADP (blue) are plotted. The empty circles on the left represent the centers of spectral mass after decompression for 15 min. (B) Root-mean-square deviation (RMSD) of Cα atoms of Hsc70 (PDB entry 1YUW) under a pressure of 3000 bar after molecular dynamics simulation for 100 ns. Hsp70 cavities (green) and voids (orange) under atmospheric pressure (C) and under a pressure of 3000 bar (D).

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caused by HHP treatment. Irreversibility was observed in both experiments in which the pressure was removed immediately or in a stepwise manner (see Experimental Procedures).

Comparing the apo curve with the ADP curve, we observed that the presence of the nucleotide enhanced the stability of Hsp70 after >1000 bar treatments (Figure 2A), suggesting that the SBD unfolds at a pressure lower than that at which the NBD unfolds. To test this hypothesis, we submitted apo-Hsp70 to pressure titration up to 1000 bar, and after equilibrium had been reached, we removed the pressure. In this case, the effects of pressure on Hsp70 were totally reversible (data not shown). Overall, we conclude that the structural changes caused by HHP treatment are irreversible but only over 1000 bar.

The mimetic peptide substrate NRLLLTG (Figure 2, filled red circle) was unable to stabilize Hsp70 against HHP, and the process was again irreversible (empty red circle).

To improve our understanding of the effects of HHP on the structure of Hsp70, we performed molecular dynamics (MD) experiments using bovine Hsc70 (PDB entry 1YUW[15]) as a model. Full-length Hsc70 was submitted to a pressure of 3000 bar for 100 ns in two independent experiments (Figure 1B). Using the root-mean-square deviation (RMSD) of Cα atoms, we observed no significant differences between the domains (Figure 2B). The cavity volumes of Hsp70 at atmospheric pressure (Figure 2C) and 3000 bar (Figure 2D) were calculated using McVol (see Experimental Procedures). We observed cavity volumes of 1579 and 358 Å^3 for the NDB and SBD, respectively, under atmospheric pressure (Figure 2C). Using the PDB entries generated from the MD simulations under 3000 bar, we calculated the ΔV of the NBD and SBD. We observed ΔV values of 593 and 170 Å^3 for the NDB and SBD, respectively. On the basis of the MD simulations, we conclude that these domains are similarly sensitive to HHP. However, on the basis of the cavity volumes and ΔV values, which were calculated using MD and McVol, it appears that the NBD is more sensitive to pressure than the SBD.

Because the changes caused by HHP treatment were irreversible according to the Trp fluorescence (Figure 2, empty circles), we characterized the species produced after one cycle of pressurization (Figure 3, green). Size-exclusion chromatography (SEC) experiments confirmed the irreversibility of the effects of pressure on the structure of Hsp70 (Figure 3A). Before pressure treatment, Hsp70 eluted as a main peak at 14 mL, which is compatible with its monomeric mass.[21] After HHP treatment, the protein eluted as a prominent peak at 8 mL (the column void), suggesting its aggregation or an expanded conformation.

The secondary structure of Hsp70 was not entirely recovered after pressure release (Figure 3B), confirming the existence of unfolded segments of the protein. We also used dynamic light scattering to estimate the hydrodynamic radius (R_h) of Hsp70 before and after HHP treatment. The R_h of Hsp70 before pressure was 3.2 nm, a value closer to that of DnaK.[7] After pressure treatment, the R_h changed to 5.4 nm, corroborating the SEC data and suggesting that Hsp70 forms oligomers or adopts an expanded conformation (Figure 3C). To determine if the enlarged R_h of Hsp70 after pressure release was due to oligomerization or an expanded conformation, we performed glutaraldehyde cross-linking experiments. The majority of Hsp70 molecules before HHP treatment (Figure 3D) were monomers (M) with a small fraction of dimers (D). However, after HHP treatment, most of the monomeric Hsp70 was converted to high-molecular weight species (oligomers, O).

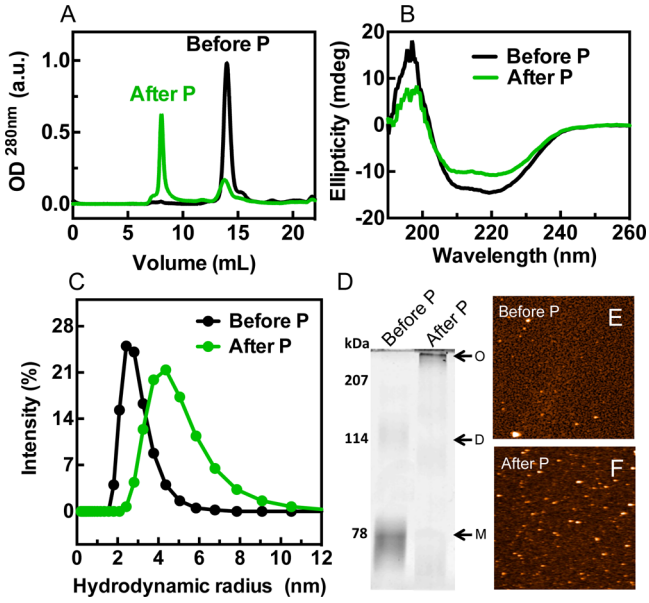

Figure 3. Hsp70 forms oligomers after HHP treatment. (A) Size-exclusion chromatography of Hsp70 before pressure treatment (P) and after decompression for 1.5 h. (B) Far-UV circular dichroism spectra of 3 μM Hsp70 before and after pressure treatment. (C) Dynamic light scattering of the samples before and after HHP treatment. (D) Hsp70 before and after HHP treatment was cross-linked with glutaraldehyde and analyzed using SDS−PAGE. The oligomers (O), dimers (D), and monomers (M) of Hsp70 were visualized by Coomassie Blue staining. Atomic force microscopy images of Hsp70 before (E) and after (F) HHP treatment. Each image is 3 μm × 3 μm.

The average volumes of Hsp70 calculated using the R_h (Figure 3C) before and after pressure treatment were 137 and 659 nm^3, respectively; assuming that Hsp70 is a perfect sphere, these data suggest that Hsp70 forms pentamers after HHP treatment. However, further structural analyses are needed to support this conclusion. Atomic force microscopy of Hsp70 before (Figure 3E) and after (Figure 3F) HHP treatment confirmed oligomerization. Therefore, after HHP treatment, Hsp70 forms oligomeric species (Figure 3A,C,D) that retain part of their original secondary structure (Figure 3B).

We then evaluated whether ATP or the mimetic peptide substrate NRLLLTG with ATP is able to dissociate the oligomers formed after HHP treatment. ATP has been shown to dissociate Hsc70 oligomers produced *in vitro*[22,23] and heat-induced aggregates of Hsc70 and Hsp70 that are observed *in vivo*.[23,24] However, neither ATP nor NRLLLTG with ATP was able to dissociate the HHP-induced oligomers of Hsp70, as assayed by glutaraldehyde cross-linking (data not shown), even when 10% native Hsp70 was added, suggesting an important difference between these oligomers and those formed by HHP treatment.

We therefore investigated whether the oligomers formed after pressure treatment retained the ability to bind and hydrolyze ATP. ATP partially protects Hsp70 against proteolysis.[24,25] Trypsin digestion revealed that the oligomers formed after HHP treatment were partially stabilized against digestion in the presence of ATP (Figure 4A). These oligomers displayed ATPase activity similar to that of native Hsp70 (Figure 4B), suggesting that the NBD of Hsp70 returns to its native folded state after pressure release and that the SBD is involved in aggregation. In this case, the chaperone activity of

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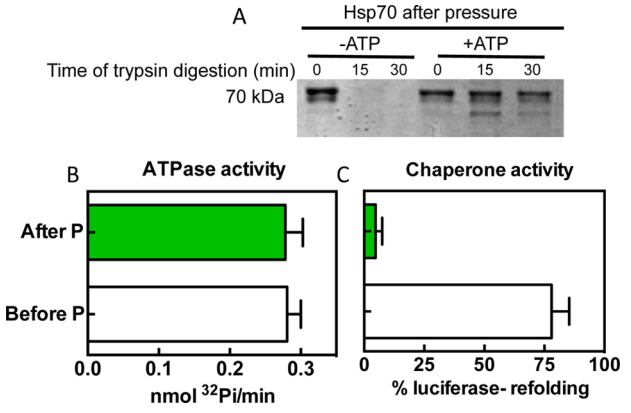

Figure 4. Oligomers produced after pressure release showed ATPase activity but lost chaperone activity. (A) Kinetics of trypsin digestion of Hsp70 after pressure treatment in the absence or presence of ATP. ATPase activity (B) and refolding of heat-denatured luciferase (C) by Hsp70 before (white) and after (green) pressure treatment. The error bars represent the standard deviation of three independent experiments.

the aggregates formed after pressure release should be lost. Indeed, oligomers of Hsp70 formed after pressure treatment were unable to refold luciferase to its native state (Figure 4C), confirming that these aggregates had lost their chaperone activity.

The results presented in Figure 4 suggest that the NBD of Hsp70 is functional but the SBD is not available to receive the substrate, in this case the unfolded luciferase. We hypothesized that after HHP treatment, the SBD of Hsp70 recognizes as a substrate one or more exposed domains of other Hsp70 molecules, leading to oligomerization (Figure 3). In fact, the crystal structure of truncated bovine Hsc70 showed that the binding site of the SBD was occupied by the partially unfolded truncated C-terminal helix of other Hsc70 molecules.[26]

Because Hsp70 has five cysteines (three on the NBD and two on the SBD), we investigated whether the presence of a reducing agent such as DTT would render the unfolding process of Hsp70 reversible and inhibit aggregation. However, even under reducing conditions, the unfolding process was irreversible and the chaperone activity of Hsp70 was completely lost after HHP treatment (data not shown). We conclude that the oligomerization induced by HHP treatment is not related to interspecies disulfide bond formation.

CONCLUSIONS

Oligomeric chaperones, such as α-crystalline, and multidomain monomeric chaperones, such as protein disulfide isomerase, can recover chaperone function after pressurization at 3000 and 4000 bar, respectively.[27,28] Here, using the monomeric multidomain chaperone Hsp70, we observed the complete inactivation of chaperone activity after pressure release. Usually, monomeric proteins are resistant to high pressure.[13] However, Hsp70 was very sensitive to HHP (Figure 2), forming soluble oligomeric species after pressure release. This observation raises the possibility that Hsp70 may depend on the activity of other chaperones to prevent the formation of similar oligomers during its folding pathway. We speculate that Hsp70 cavities make this chaperone inherently pressure sensitive and may be important for the conformational changes that are a part of Hsp70 function.

While we could not determine which domain is more sensitive to pressure (Figure 2), it was clear that NBD can return to its native state (Figure 4), whereas the SBD remains misfolded or inaccessible to the substrate after HHP release.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: palhano@bioqmed.ufrj.br. Phone or fax: (+55) 021 3938-6761.

*E-mail: foguel@bioqmed.ufrj.br.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

HHP, high hydrostatic pressure; NBD, nucleotide binding domain; SEC, size-exclusion chromatography; SBD, substrate binding domain.

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