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Stoichiometry and thermodynamics of the interaction between the C-terminus of human 90 kDa heat shock protein Hsp90 and the mitochondrial translocase of outer membrane Tom70

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ABSTRACT

A large majority of the 1000–1500 proteins in the mitochondria are encoded by the nuclear genome, and therefore, they are translated in the cytosol in the form and contain signals to enable the import of proteins into the organelle. The TOM complex is the major translocase of the outer membrane responsible for preprotein translocation. It consists of a general import pore complex and two membrane import receptors, Tom20 and Tom70. Tom70 contains a characteristic TPR domain, which is a docking site for the Hsp70 and Hsp90 chaperones. These chaperones are involved in protecting cytosolic preproteins from aggregation and then in delivering them to the TOM complex. Although highly significant, many aspects of the interaction between Tom70 and Hsp90 are still uncertain. Thus, we used biophysical tools to study the interaction between the C-terminal domain of Hsp90 (C-Hsp90), which contains the EEVD motif that binds to TPR domains, and the cytosolic fragment of Tom70. The results indicate a stoichiometry of binding of one monomer of Tom70 per dimer of C-Hsp90 with a K_D of 360 ± 30 nM, and the stoichiometry and thermodynamic parameters obtained suggested that Tom70 presents a different mechanism of interaction with Hsp90 when compared with other TPR proteins investigated.

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Introduction

Mitochondria, which are ubiquitous in eukaryotic cells types, are crucial to many physiological functions [1,2], and they have critical roles in physiological and pathological processes and act as cellular "powerhouses". In addition to being vital for energy production and therefore for the survival of eukaryotic cells, mitochondria are implicated in a number of other essential functions, such as cellular respiration, metabolism of lipids, amino acids, iron homeostasis, and the regulation of intrinsic pathway of apoptosis [3–5].

Mitochondria are double-membrane bound organelles that include four compartments: the outer membrane, the inner membrane, the intermembrane space and the mitochondrial matrix. In the interior of the mitochondria, about 1000–1500 different proteins are present, of which only a small fraction are encoded by the mitochondrial genomic DNA [4,6]. The vast majority of mitochondrial proteins are encoded by the nuclear genome, translated by cytosolic ribosomes in the form of preproteins and thereafter translocated into the mitochondria [6–8]. Preproteins targeted specifically to the mitochondria contain signals to localize to the correct compartments within the organelle. The signals are recognized by cytosol-exposed receptors located in the mitochondrial outer membrane [4]. Following recognition, these signals are decoded by mitochondrial translocases, which contain multifunctional components that coordinate the preprotein transfer to the translocation pore and that control the sorting and communication with subsequent translocases [1,6,8]. Thus, the machineries of importing and sorting proteins into the mitochondria are greatly important in maintaining proper mitochondrial function.

A major translocase of the outer membrane is the TOM complex. It consists of the general import pore (GIP) complex and two membrane import receptors, Tom20 and Tom70, with a single N-terminal transmembrane domain and a receptor domain exposed into the cytosol [5,8]. Tom20 recognizes the N-terminal mitochondrial targeting signals from the preproteins, via hydrophobic interactions, while Tom70 preferentially recognizes preproteins with internal hydrophobic targeting sequences, such as the inner membrane metabolite carriers [4,5,9–12]. The Tom70-mediated import pathway is chaperone dependent. Molecular chaperones Hsp70 and Hsp90 have important roles in targeting the preproteins to the TOM complex and in the protection of these

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preproteins from aggregation in the cytosol [13]. Tom70 contains a cytosolic TPR (tetratricopeptide repeat) clamp domain that serves as a docking site for the Hsp70 and Hsp90 C-terminal EEVD motifs and consequently for the multi-chaperone complexes that hold the preprotein. The C-terminal EEVD motif of the chaperones has been implicated in the binding of other proteins containing TPR regions, including Hop and CHIP co-chaperones [14–18]. The ATPase cycles of Hsp70/Hsp90 control interactions with substrates and client proteins and are also needed for the chaperones to assist in protein folding; in addition, preproteins are transferred in an ATP-dependent way to the TOM complex for translocation [19]. Specific Hsp90 inhibitors reduce the Tom-dependent import to a basal level, indicating that the chaperone has a significant role in protein import into the mitochondria via the TOM complex [19]; however, many aspects of the Tom70–Hsp90 interaction are still not clear. For instance, the details of the mechanism of interaction and the structure of the complex remain unclear. In a previous work, we showed that the functional state of human Tom70 is monomeric [5], and here, we present the stoichiometry and thermodynamic parameters of its interaction with the C-terminal domain of Hsp90, which is a dimer in solution.

Materials and methods

Plasmids, recombinant protein expression and purification

The coding sequences for the C-terminus fragment of human Hsp90α, C-Hsp90, (NCBI Accession No.: AAI21063, residues 566–732) and the cytosolic fragment of human Tom70 (NCBI Accession No.: O94826.1, residues 111–608) cloned into a pProExHta (Invitrogen), which inserts a His-tag followed by a cleavage site for the TEV-protease, were a generous gift from Dr. Jason C. Young [14]. Both recombinant proteins were expressed in BL21(DE3) *Escherichia coli* cells and were induced with 0.8 mM isopropyl β-D-thiogalactoside at 37 °C for 4 h. His-tagged proteins were purified using a nickel–Sepharose 5 mL column (GE Healthcare) equilibrated in 20 mM sodium phosphate, pH 7.4, 500 mM NaCl and 20 mM imidazole, and they were eluted with 20 mM sodium phosphate, pH 7.4, 500 mM NaCl and 150 mM imidazole. Proteins were further purified over a Superdex 200 26/60 gel filtration column (GE Healthcare) in 20 mM Tris–HCl, pH 8.0, 150 mM NaCl using an ÄKTA FPLC instrument (GE). Protein purification was assessed by SDS–PAGE, and concentrations were determined using the absorbance at 280 nm.

Spectroscopic and hydrodynamic experiments of C-Hsp90

Circular dichroism (CD)[1] measurements were recorded on a Jasco J-810 spectropolarimeter (JASCO, Inc.) with temperature controlled by a Peltier-type system (PFD 425S). Data were collected at 20 °C using a 1 mm pathlength cuvette, in 20 mM Tris–HCl, pH 8.0, and 150 mM NaCl with a protein concentration of 10 μM. Fluorescence measurements were done in a SLM AMINCO-Bowman Series 2 (AB2) spectrofluorimeter (Thermo Fisher Scientific, Inc.) using a 1 × 1 cm pathlength cuvette with 10 μM of C-Hsp90, in the same buffer as for the CD experiments. Excitation was at 295 nm and emission was measured from 300 to 420 nm (emission and excitation bandpass of 4 nm). Fluorescence data were evaluated by their emission maxima wavelength (λ_{max}) and by their spectral center of mass ($\langle\lambda\rangle$), as described by the equation:

$$\langle\lambda\rangle = \frac{\sum \lambda_i F_i}{\sum F_i} \quad (1)$$

where λ_i is each wavelength and F_i is the fluorescence intensity at λ_1.

Analytical ultracentrifugation experiments were conducted with a Beckman Optima XL-A analytical ultracentrifuge and an AN-60Ti rotor (for review, see [20]). Sedimentation equilibrium (SE) experiments were performed at protein concentrations from 200 to 600 μg/mL (approximately 5–15 μM of dimeric C-Hsp90), in a buffer containing 20 mM Tris–HCl, pH 8.0, and 150 mM NaCl at 20 °C, from 9000 to 13,000 rpm, and data acquisition was at 276 nm. The software SEDPHAT version 8.2 was applied to evaluate the SE data [21]. The partial specific volume of the protein, buffer density and viscosity were calculated with the program SEDNTERP [22]. The global fitting was accomplished with the "Species Analysis" model of the SEDPHAT program. All parameters were allowed to float freely, and then, the statistical analyses were performed. The statistical method used was the "Monte-Carlo non-linear regression" with at least 100 iterations and a confidence level of 0.68. Tom70 was similarly analyzed by CD, and fluorescence spectroscopy and analytical ultracentrifugation were performed as described in a previous work by some of the authors [5].

Pull-down experiments

The experiments were conducted at room temperature. The His-tag of C-Hsp90 was removed by the TEV-protease so that C-Hsp90 could be used as the prey in the pull-down assays, while the His-tag of Tom70 was maintained to be used as the bait. Pull-down experiments were performed in equilibrium buffer (50 mM sodium phosphate, 300 mM NaCl, pH 8.0), with 50 μM of His_6–Tom70 and 25 μM (dimer concentration) of cleaved C-Hsp90. Proteins were mixed and incubated for 2 h, and then, 100 μL of nickel–agarose resin Ni–NTA (Qiagen) was added, and the mixture was further incubated for 2 h. The resin was washed four times with 200 μL aliquots of equilibrium buffer added to 20 mM imidazole to release proteins bound nonspecifically to the resin. Proteins bound specifically to the resin and any proteins interacting with them were eluted with the equilibrium buffer added to 200 mM imidazole, precipitated with acetone, solubilized in sample buffer and analyzed by Coomassie-stained SDS–PAGE.

Size-exclusion chromatography–multiple angle light scattering (SEC–MALS)

The oligomeric states of Tom70, C-Hsp90 and their complex were estimated via SEC–MALS measurements on an ÄKTA FPLC system (GE Healthcare) connected to a triple-angle static light-scattering detector miniDAWN™ TREOS (Wyatt Technology, Santa Barbara, CA, USA). A Superdex 200 HR 10/300 GL column (GE Healthcare) was used in 20 mM Tris–HCl, pH 8.0, and 150 mM NaCl at a flow rate of 0.5 mL/min. Sample volumes of 250 μL were injected at a concentration of approximately 100 μM of Tom70 and 50 μM of C-Hsp90 (dimer concentration). The data were recorded and processed using ASTRA V software (Wyatt Technology, Santa Barbara, CA, USA). To determine the detector delay volumes and normalization coefficients for the light scattering detector, a BSA sample (Sigma–Aldrich®) was used as a reference. SEC–MALS is an absolute method for molecular mass determination; it provides a direct measure of molecular mass without being limited by the molecular shape or hydrodynamic parameters. The signal from the light-scattering detector is directly proportional to the molecular mass of the protein multiplied by their concentrations (mg/mL). This signal and the concentration (determined by absorbance, for example) make it possible to measure the molecular

[1] *Abbreviations used:* C-Hsp90, Hsp90 C-terminal domain; CD, circular dichroism; ITC, isothermal titration calorimetry; K_D, dissociation constant; SEC-MALS, size-exclusion chromatography coupled to multi-angle light scattering; SE, sedimentation equilibrium; MM, molecular mass; Tom70, translocase of the outer membrane.

mass of each peak eluting from the column [23], so it is a reliable technique to determine the molecular mass and stoichiometry of protein–protein complexes. All elution peaks were collected in 0.5 mL aliquots and were assessed by Coomassie-stained SDS–PAGE.

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were performed at 20 °C using a VP-ITC calorimeter (Microcal, LLC, Northampton, MA, USA) running Origin version 5.0 software. All solutions were thoroughly degassed before use by stirring under vacuum, and protein samples were dialyzed against 20 mM Tris–HCl, pH 8.0, and 150 mM NaCl. Tris buffer was used to facilitate the comparison with previous works ([25] and references therein) and to eliminate possible artifactual results caused by the interaction of phosphate with a putative additional nucleotide binding site in the C-terminus of Hsp90 [25] (see also Results and discussion). Titrations consisted of 5 μL injections of 109 μM Tom70 every 300 s into 5 μM C-Hsp90 solutions (dimer concentration). The enthalpy change for each injection was calculated by integrating the area under the peaks of the recorded time course of the change of power and then by subtracting that of the control titration. The control experiments were used to correct for heat effects of dilution and mixing, and they consisted of injections of buffer without protein into buffer. The heat of dilution was determined from the baseline at the end of titration and was negligible. The apparent enthalpy change of binding (ΔH_{app}, i.e. the sum of all events involved in the interaction), binding stoichiometry (n), and dissociation constant (K_D) were estimated from the best fit of the theoretical titration curve using a least squares fitting analysis (Origin 5.0).

Results and discussion

The C-terminus of Hsp90 was produced folded as a dimer in solution

The 90 kDa heat shock protein Hsp90 is one of the most important molecular chaperones, and it has only one homolog in prokaryotes, whereas in humans there are at least four homologs, two cytosolic, one in the mitochondria and one in the endoplasmic reticulum. Hsp90 is essential for survival in eukaryotes, and its client proteins are normally associated with cell cycling and signaling, and this association makes this chaperone a potential drug target [26–28]. Hsp90 is an elongated dimer in solution with the dimerization site located in the C-terminal domain [29–31], and it can be divided into specific domains, the N-terminal domain followed by a linker, the M or middle domain, and the C-terminal domain, which has the EEVD motif that is involved in the binding to proteins presenting the TPR domain. In the case of the inducible cytosolic Hsp90α, the N-terminal domain comprises residues 1–210, the linker comprises residues 211–272, the M or middle domain comprises residues 273–629, and the C-terminal domain comprises residues 630–732 [26]. The EEVD motif is located at residues 729–732 and is essential for Hsp90 binding to its co-chaperone by the TPR domain present in these proteins. To gain insight into the mechanism by which Hsp90 interacts with Tom70, we purified a recombinant C-terminus fragment of human Hsp90α (C-Hsp90) using a modified approach and characterized its folded state.

C-Hsp90 was pure and soluble (data not shown). Its CD spectrum is characteristic of a folded protein [32] with minima at 209 nm ($-10{,}840$ deg cm^2 dmol^{-1}) and 222 nm ($-10{,}670$ deg cm^2 dmol^{-1}) as shown in Fig. 1A. By applying the simple prediction methods proposed by Morriset et al. [33] and Greenfield and Fasman [34], the percentage of α-helix and β-sheet were estimated to be of approximately 26% and 37%, respectively. These percentages are in good agreement with the crystal structure of the C-terminus of the Hsp90 homolog from *Saccharomyces cerevisiae* (PDB ID: 2CG9), in which the C-terminus comprises a three-stranded β-sheet positioned under a curved helix that faces a three-helix coil [35]. Emission fluorescence spectroscopy of tryptophan (Trp) is a reasonable method to access information regarding the environment where this residue is situated in a protein, because of its sensitivity to the polarity of this environment; thus, Trp is a local probe of the folded state of a protein [36]. C-Hsp90 has one Trp residue. The emission fluorescence spectrum had its maximum intensity at 343 ± 1 nm with a spectral center of mass at 341 ± 1 nm (Fig. 1B), suggesting that the residue was well-buried in the protein structure. This result is another indication that the protein was pure.

Sedimentation equilibrium analytical ultracentrifugation was used to determine the molecular mass (MM) and thus the oligo-

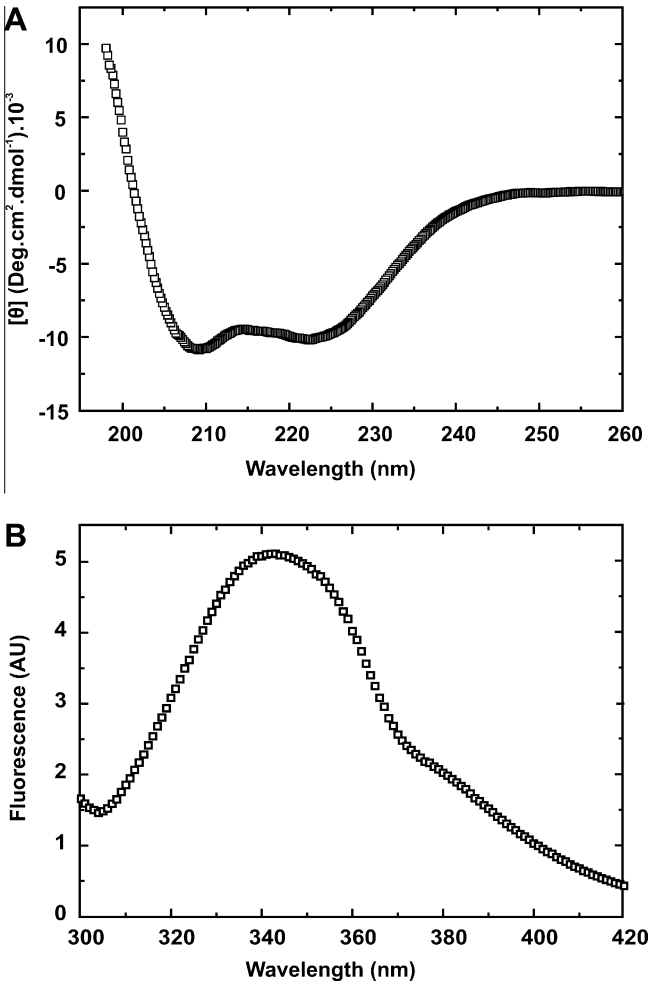

Fig. 1. (A) Circular dichroism spectrum of recombinant human C-Hsp90. Mean residue ellipticity ([θ]) was measured from 198 to 260 nm at a protein concentration of 10 μM, in 20 mM Tris–HCl, pH 8.0, with 150 mM NaCl at 20 °C, using a Jasco J-810 spectropolarimeter. The spectrum indicates that C-Hsp90 is folded and, by applying simple methods proposed by Morriset et al. [33] and Greenfield and Fasman [34], has about 26% and 37% α-helix and β-sheet content, respectively. (B) Emitted fluorescence spectrum of recombinant human C-Hsp90. Emission tryptophan fluorescence was measured from 300 to 420 nm; the excitation was at 295 nm at a protein concentration of 10 μM, using a SLM AMINCO-Bowman Series 2 (AB2) spectrofluorimeter. C-Hsp90 has one Trp residue, and the emission fluorescence spectrum had a maximum intensity at 343 ± 1 nm with a spectral center of mass at 341 ± 1 nm suggesting that the residue was well-buried in the protein structure.

meric state of C-Hsp90. The results of three different protein concentrations at five different speeds were analyzed globally with SEDPHAT (see Materials and methods). Fig. 2 shows the results using 600 μg/mL protein with the residuals from fitting shown in the upper panel. As determined by the fitting and the Monte-Carlo statistics analysis, C-Hsp90 has a molecular mass of 43 ± 1 kDa, which is consistent with a dimer in solution, since the MM predicted from the primary structure for the recombinant monomer is 21850.75 Da. Taken together, our results indicate that C-Hsp90 is properly folded and is a dimer.

Stoichiometry and thermodynamics of the interaction between C-Hsp90 and Tom70

The characterization of the folded conformation and oligomeric state of the recombinant cytosolic fragment of human Tom70 has been described previously [5], where we and our collaborators showed that the protein is purified in its natively folded state, has high α-helix content and is a functional monomer. Since both C-Hsp90 and Tom70 are well-behaved single species in solution, we undertook the measurement of their interaction. The interaction was initially studied by pull-down assays. For that examination, the Tom70 His-tagged construct was maintained intact to be used as bait in pull-down assays, since it was capable of binding to a nickel resin, whereas C-Hsp90 had it His-tag cleaved by TEV-protease. The results were assessed by PAGE in denaturing conditions by SDS (Fig. 3). The experiment showed that His-tagged Tom70 bound the resin as expected and free C-Hsp90, which has the His-tag removed did not (Fig. 3). However, when mixed with Tom70, C-Hsp90 remained bound to the resin even after two washes and was released only when imidazole was used to decrease the affinity of His-tagged Tom70 for the resin (Fig. 3). Therefore, these results demonstrate that C-Hsp90 in fact interacted with Tom70. We then set out to determine the oligomeric state of the proteins in the complex by measuring the MM of the complex. We used size-exclusion chromatography combined with multi-angle static light scattering (SEC–MALS), a technique that provides a precise determination of MM independent of molecular shape or hydrodynamic parameters, to determine the MM of the complex (Fig. 4). Fig. 4A shows the SEC–MALS measurements of isolated proteins as normalized light scattering profiles and respective molecular mass distributions. Fig. 4B and C show the SDS–PAGE profiles of free Tom70 and free C-Hsp90 (B) and their mixture (C) from SEC–MALS, to confirm the presence of the proteins. As expected, free C-Hsp90 had a MM of ~43 kDa (Fig. 4A and B), which is in excellent agreement with the theoretically calculated MM of 43.7 kDa for a dimer, corroborating SE results (see above), and free Tom70 had a MM of ~62 kDa (Fig. 4A and B), consistent with the theoretical calculated MM of 59.7 kDa for a monomer, which is supported by previously analysis of its oligomeric state in solution [5]. Mixed Tom70 and C-Hsp90 eluted as a single peak with a MM of ~110 kDa (Fig. 4A and C), indicating a stoichiometry of one monomer of Tom70 to a dimer of C-Hsp90 in the complex.

The stoichiometry determined above by SEC–MALS was confirmed by isothermal titration calorimetry (ITC). In this experiment, the ΔH_{app} for the binding interaction is measured at a constant temperature, allowing for the determination of the binding stoichiometry (n) and the dissociation constant (K_D) by fitting of the titration curve. Both the sequence of injections and the differential heat plot are shown in Fig. 5. In accordance with the conclusion from the SEC–MALS results, the binding stoichiometry (n) was equal to 1.2 ± 0.1, indicating that one monomer of Tom70 binds to a dimer of C-Hsp90. The K_D was determined to be 360 ± 30 nM, suggesting that the complex has high affinity. The ΔH_{app} of interaction was −2.6 ± 0.1 kcal/mol, and the apparent entropy of interaction was 21 ± 1 cal/mol K, indicating that the interaction is driven by both enthalpy and entropy. Interestingly, Hop and Cyp40, other TPR co-chaperones from human, bind the

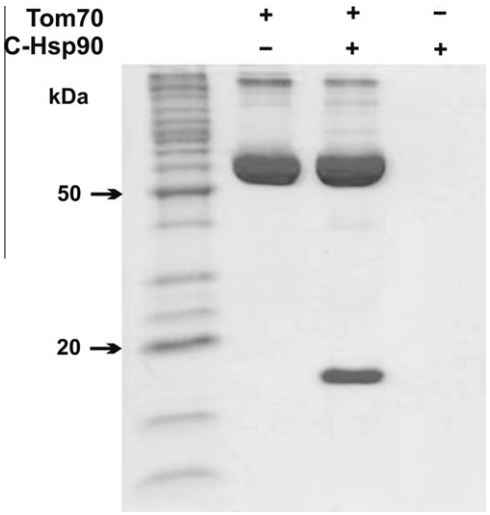

Fig. 3. Pull-down assays of Tom70 and C-Hsp90. The Tom70 His-tagged construct was maintained intact whereas C-Hps90 had its His-tag cleaved by TEV-protease to be used as bait in pull-down assays. SDS–PAGE analysis of samples bound to the Ni–NTA resin that eluted in the presence of 200 mM imidazole (see Materials and methods). Lane 1, molecular mass marker (BenchMark™ Protein Ladder from Invitrogen); lane 2, Tom70 used as control; lane 3, Tom70 C-Hsp90 mixture; and lane 4, C-Hsp90 used as control. His-tagged Tom70 was capable of binding to C-Hsp90 and pulled it out of solution, confirming their interaction (lane 3).

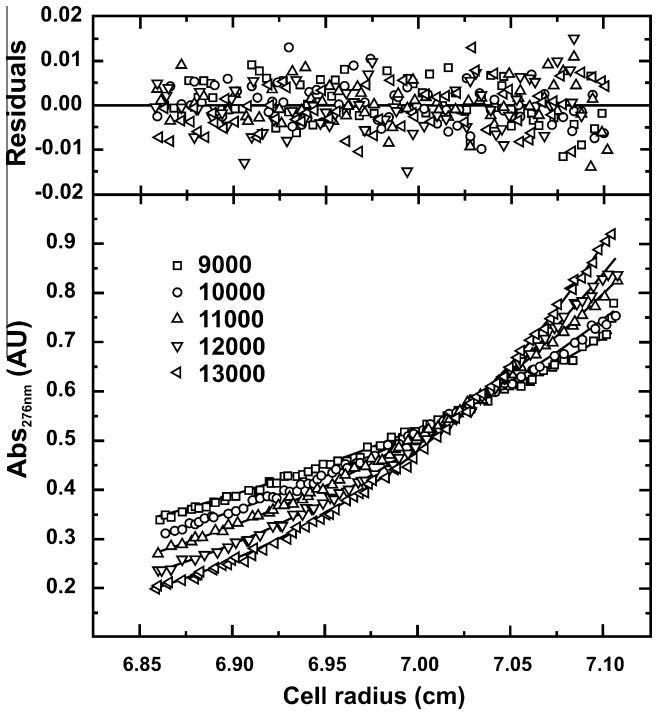

Fig. 2. Analytical ultracentrifugation sedimentation equilibrium of C-Hsp90. SE experiments were made at protein concentrations from 200 to 600 μg/mL (approximately 5–15 μM of dimeric C-Hsp90), in 20 mM Tris–HCl, pH 8.0, and 150 mM NaCl at 20 °C, from 9000 to 13,000 rpm, and data were acquired at 276 nm. The data analysis was performed with the software SEDPHAT version 8.2, and hydrodynamic parameters were calculated with SEDNTERP. Global reduced χ^2 was approximately 1.07, and the local rmsd was approximately 0.0052. The fitting and Monte-Carlo statistics indicated that C-Hsp90 is a dimer in solution, with a molecular mass of 43 ± 1 kDa, consistent with the known data for full-length Hsp90.

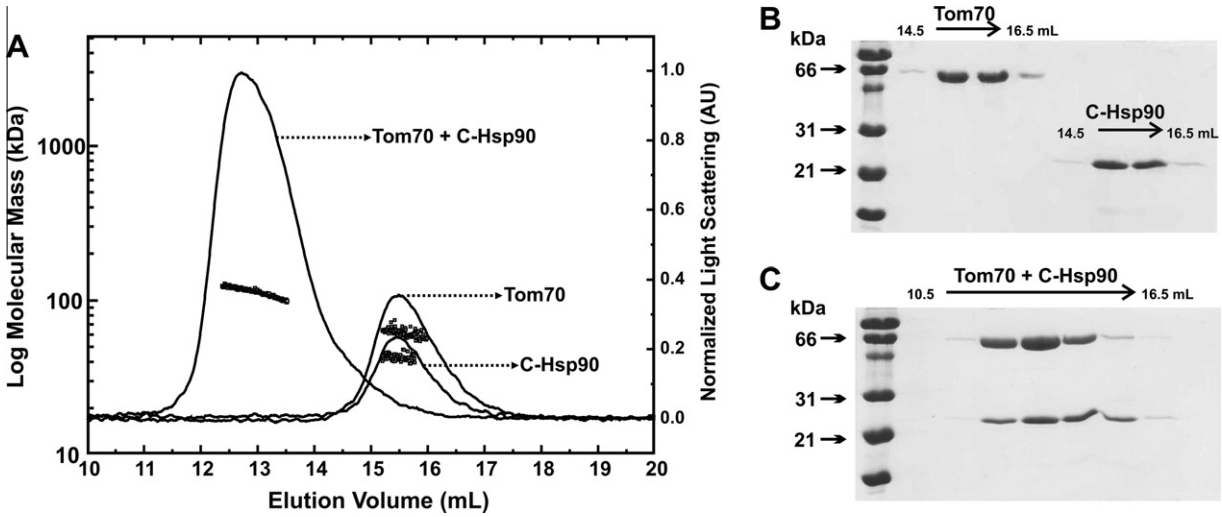

Fig. 4. SEC–MALS combined with SDS–PAGE for the determination of the molecular mass of the Tom70 C-Hsp90 complex. (A) SEC–MALS measurements of the isolated proteins and the complex are labeled. The graphic shows the representative normalized light scattering profiles recorded by detectors (continuous lines) and the molecular mass distributions (open squares) per elution volume unit (mL). Free C-Hps90 had a MM of ~43 kDa (consistent with a dimer), free Tom70 had a MM of ~62 kDa (consistent with a monomer) and mixed Tom70 C-Hsp90 had a MM of ~110 kDa, indicating a stoichiometry of one monomer of Tom70 to a dimer of C-Hsp90 in the complex. (B and C) Fractions of 0.5 mL from free Tom70 and free C-Hsp90 (B) and complex (C) were collected and assessed by SDS–PAGE to confirm the presence of the proteins in the SEC–MALS measurements.

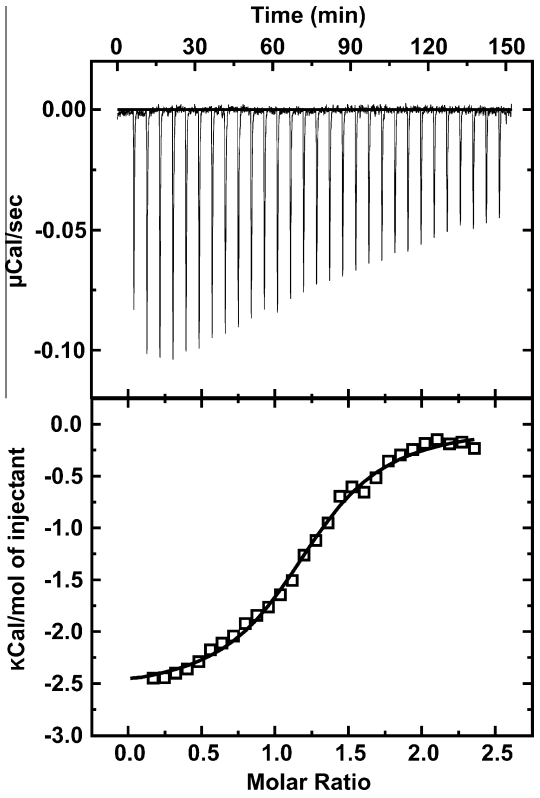

Fig. 5. Binding of Tom70 to C-Hsp90 monitored by ITC. Top, the experimental outline of the injections of Tom70 into the C-Hsp90 dimer showing thermal power, which is proportional to the heat of each injection, as a function of time. Bottom, the integrated heat plot. Heats of binding are represented by open squares, and the line represents the best fit of the data using the Origin 5.0 (Microcal). The binding stoichiometry (n) was equal to 1.2 ± 0.1, indicating that one monomer of Tom70 binds to a dimer of C-Hsp90. Data analysis provided a K_D value of 360 ± 30 nM, suggesting that the complex has a high affinity. The binding ΔH measured from a single ITC experiment might include heat that is due to buffer ionization; therefore, the ΔH measured here is referred to as ΔH_{app} and was −2.6 ± 0.1 kcal/mol. The apparent entropy of interaction was 21 ± 1 cal/mol K indicating that the interaction is driven by both enthalpy and entropy.

C-terminus of human Hsp90-β with a K_D of about 10 times greater than that of Tom70, in an interaction that is also enthalpy driven, although the interaction with Cyp40 is driven by entropy [17,24]. It may be possible that there are slightly differences in the binding of different TPR co-chaperones with Hsp90. There are crystal structures of the Hsp90 MEEVD motif in complex with the TPR domains of human Hop [17] and yeast Tom 71 [4] (Fig. 6). Since the residues of Tom71 that interact with the MEEVD motif are conserved in human Tom70, the structure of the yeast ortholog can be used as a model for the interaction of Tom70 with Hsp90 [4]. First, one can observe general similarities. The interaction of the MEEVD with the TPR domains of both Hop and Tom71 involves mainly the dicarboxylate from the carboxy-terminal Asp residue from MEEVD and two basic lysines from the TPR domain (Fig. 6). At pH 8, the two lysines are positively charged while the MEEVD motif is negatively charged showing that the interaction between TPR co-chaperones and Hsp90 is electrostatic in nature. Second, slightly differences in binding can be observed when comparing Fig. 6A with B and C with D. The MEEVD motif appears to be more involved by the TPR domain when bound to Hop than when bound to Tom71, suggesting that the interaction between the MEEVD and Hop is more organized and thus has a higher entropic cost. In addition to that, the conformation of the MEEVD pentapeptide changes depending on the TPR co-chaperone to which it is bound (Fig. 6). Six residues from the Tom71 TPR domain are involved in seven electrostatic interactions with the MEEVD motif while seven residues from the Hop TPR domain are involved in ten electrostatic interactions with the MEEVD motif (Fig. 6A and B). The seventh residue is an Asn in position 308 of the TPR2a from Hop that has no equivalent in Tom71 and is hydrogen bonded to the first Glu residue of the MEEVD motif. A Tyr at position 236 of the TPR2a from Hop is hydrogen bonded to the carbonyl group of the peptide bond between the two Glu residues of the MEEVD motif (Fig. 6B), while the correspondent residue in Tom71, Phe138, does not make this kind of interaction (Fig. 6A). In contrast with the tight interactions when bound to Hop, the N-terminus of the MEEVD motif appears to be less restrained when bound to Tom71 (Fig. 6), indicating that although the interactions of this portion of the motif with Hop increase enthalpy they also have an entropic cost due to the organization of the complex. This observation is

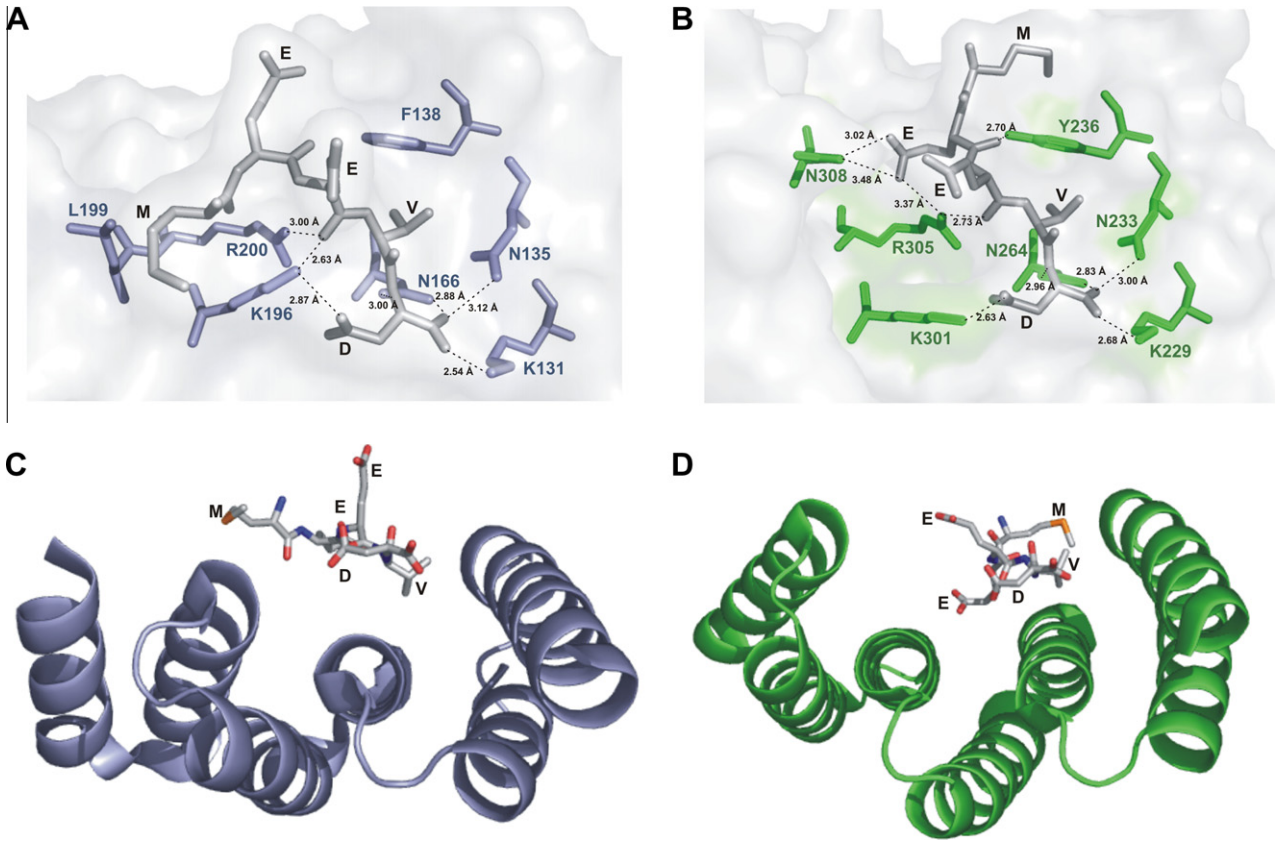

Fig. 6. The MEEVD motif from Hsp90 complexed with TPR domains. The structures of the MEEVD in complex with the TPR domains of yeast Tom71 (A and C) – PDB 3FP2; and human Hop (B and D) – PDB 1ELR) are shown. Electrostatic interactions and hydrogen bounds are highlighted in panels (A) and (B) while panels (C) and (D) present the front view of the complexes, highlighting the relative position of the dicarboxylate clamp (see text). Generated using Pymol (www.pymol.org).

supported by ITC experiments that measured the interaction of C-Hsp90 with Hop [24] and were performed in similar conditions to those reported in this work. Onuoha et al. [24] showed that the interaction between C-Hsp90 and Hop has a higher enthalpic contribution than that measured for Tom70 (this work) and a negative entropy value which is different from the interaction of C-Hsp90 with Tom70 that was entropy driven (this work). Additionally, when bound to the TPR domain of Tom71, the Met residue of the MEEVD motif makes hydrophobic interactions with a cleft formed by the protein residues Leu199 and Lys196 (Fig. 6A), indicating that the binding may have a lower apparent enthalpy of binding but also a positive entropic contribution. This observation is also in good agreement with our measurements on human Tom70 and is expected since hydrophobic interactions provide a higher degree of freedom.

Finally, the stoichiometry of binding for either Hop or Cyp40 is of one monomer per monomer of C-Hsp90-β [17,24], although some controversy exists regarding the oligomeric state of native Hop [37,38]. Therefore, the mode of interaction between Tom70 and C-Hsp90 appears to have specific characteristics that differ from those of other proteins containing TPR domains. Combined, these results suggest that the interaction of TPR proteins with Hsps may be more complex and diverse than previously expected. Moreover, it may be possible that the interaction with only one monomer of the C-Hsp90 motif allows the other to be available for additional interactions with other TPR co-chaperones, opening up the possibility for further functional modulation. However, more experimental evidence is necessary to support these hypotheses. Nevertheless, it is noteworthy that Tom70 also binds Hsp70, which is a monomer in solution [39,40].

These findings are potentially relevant because TPR–Hsp complexes have been a target of several investigations because of their potential as drug targets. Since Hsp90 is involved with the stabilization of the tumor phenotype, the interference with the interaction between the EEVD motif and TPR may inhibit the delivery of client proteins to Hsp90 [27]. Many hybrid peptides that mimic TPR domains are under investigation to substitute the general broad effect of small molecule inhibitors by a more specific inhibition strategy that will interfere with the interaction of Hsp90 with regulatory co-chaperones. We believe that the results presented here are an important step toward the design of such therapeutic strategies, and thus, they are of potential interest not only for those studying domain–domain interactions but also for those in the field of translational medicine.

Conclusion

Experiments using circular dichroism and intrinsic fluorescence spectroscopy were consistent with the purification of folded recombinant human proteins. Analytical sedimentation equilibrium showed that C-Hsp90 is a dimer in solution, a result corroborated by SEC–MALS experiments. As previously reported [5], Tom70 is a monomer in solution and interacts with C-Hsp90 forming a complex in which one monomer of Tom70 binds a dimer of C-Hsp90. The stoichiometry was confirmed by isothermal titration calorimetry, which also yielded the thermodynamic parameters of the interaction showing that human Tom70 has a high affinity for C-Hsp90 in comparison to other TPR proteins. The mode of binding between Tom70 and C-Hsp90 provides new information when

compared with what is currently known for the binding of other proteins that contain TPR domains. This information is potentially relevant for studies aiming to interfere with the interaction between the EEVD motif and TPR as this strategy may inhibit the delivery of client proteins to Hsp90.

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