Re-evaluating the Conformation Equilibrium of Grp94 Molecular Chaperone

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Abstract

Hsp90 is a highly conserved family of dimeric molecular chaperones whose function depends on ATP binding and hydrolysis. Metazoans express three classes of Hsp90: cytosol specific (e.g. Hsp82 in yeast); mitochondria specific (Trap1); and endoplasmic reticulum specific (Grp94). Although these three Hsp90 classes all share a common dependence on ATP, there is conflicting structural information about their ATP-driven conformational cycle. For example, non-hydrolysable ATP (AMPPNP) causes Trap1 and Hsp82 to undergo an open to closed conformational change, however Trap1 has an asymmetric closed structure whereas Hsp82 is symmetric. The Hsp82 structure was determined with a symmetrically bound inhibiting cochaperone, suggesting the possibility that the symmetric closed state is inactive. Adding further confusion, a structure of Grp94 has been reported with AMPPNP but in an open conformation, in marked contrast to the results from other Hsp90 homologs. These divergent structural conclusions make it difficult to propose a common mechanism. Here we revisit previous structural work on Grp94. We find that a kinetic trapping strategy can be used to accumulate a uniform closed population of Grp94. Small angle x-ray scattering measurements and computational modeling reveals that the closed state of Grp94 is well described by a symmetric conformation. To critically evaluate this structural conclusion, we constructed a model of closed symmetric Grp94 and used this model to rationally design ATPase activating mutations that remove hydrophobic surface area selectively from the closed state. Similarly, based on our closed state model, we identified a salt bridge mutation that also activates Grp94. Our results clarify conflicting structural conclusions concerning Grp94, and provide a rational engineering strategy for increasing the activity of any Hsp90.
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Introduction

The heat shock protein 90 (Hsp90) family of molecular chaperones perform essential functions by maintaining the correct folding of cellular, membrane and secreted proteins\textsuperscript{1–3}. Metazoans express specific Hsp90 proteins in the cytosol, the mitochondria (Trap1) and the endoplasmic reticulum (Grp94). The biological function of Hsp90 is dependent on ATP binding and hydrolysis\textsuperscript{4,5}. Many “client proteins”, which rely on Hsp90 function, are involved in signal transduction\textsuperscript{6–9}, cellular transportation\textsuperscript{3,10,11}, and protein degradation\textsuperscript{12–14}. Hsp90-specific inhibitors, such as radicicol, are being tested as a treatment for a wide variety of cancers\textsuperscript{15}. However, the mechanism by which Hsp90 utilizes ATP chemical energy to perform its numerous functions, is not understood.

Hsp90 is a dimer, and each the monomer is composed of three domains: the N-terminal domain (NTD), middle domain (MD) and C-terminal dimerization domain (CTD). Hsp90 adopts a wide variety of conformational states via rigid-body movements of these individual domains\textsuperscript{16–18}. Hsp90 can adopt a structurally heterogeneous open, catalytically inactive, state in which ATP can bind at the NTD. Small angle x-ray scattering (SAXS) measurements show that the open state heterogeneity largely results from MD/CTD rearrangements (Fig. 1A, double arrows)\textsuperscript{17,19}. Closure of the dimer arms is necessary for ATP hydrolysis\textsuperscript{20}, and is rate-limiting for the ATPase activity\textsuperscript{21,22}. FRET measurements on Hsp90 have shown that closure is slow, implying a large energy barrier separating the open and closed configurations\textsuperscript{22}. The closure rate of Hsp90 is likely important to its function as this rate can be regulated by client binding and cochaperones\textsuperscript{23–30}. However, the structural and energetic basis of this large closure energy barrier is not yet understood.
One impediment to understanding Hsp90 activity is that two dramatically different closed conformations have been reported. The yeast cytoplasmic Hsp90 (Hsp82) closed structure is symmetric, whereas the zebrafish mitochondrial Hsp90 (Trap1) closed structure is asymmetric (Fig. 1B). The Trap1 asymmetry is most pronounced at the MD/CTD interface (Fig. 1B, asterisk). The functional relevance of these different closed conformations is not yet clear. Importantly, the Hsp82 closed structure was determined with a symmetrically positioned cochaperone, p23, which acts as an ATPase inhibitor. This has raised the possibility that the symmetric closed conformation may not be populated in the normal Hsp90 ATP-driven conformational cycle. Additional structural and mechanistic experiments are needed to determine whether other Hsp90 homologs adopt a symmetric or asymmetric closed structure. However, structural work on any Hsp90 is challenging due to the extreme levels of conformational heterogeneity.

**Figure 1.** Hsp90 conformational changes induced by AMPPNP. (A) Trap1 adopts an asymmetric closed conformation, while Hsp82 adopts a symmetric closed conformation. (B) Close inspection of the side-views of Trap1 and Hsp82, indicate that the TRAP1 asymmetry originates at the interface between the middle and C-terminal domains (asterisk). (C) Surprisingly, the full-length Grp94 crystal structure suggests a stable open conformation in the presence of AMPPNP.
Grp94 illustrates the technical difficulties associated with Hsp90 conformational heterogeneity. Specifically, a full-length Grp94 structure\textsuperscript{33} has been determined with adenosine 5'-(β,γ-imido) triphosphate (AMPPNP) but the structure is not closed. The preference for the Grp94 open configuration has been further suggested by SAXS and electron microscopy (EM) measurements, which indicated only a small compaction of Grp94 in the presence of the non-hydrolyzing ATP analog, AMPPNP\textsuperscript{34}. Thus Grp94 may operate by a different mechanism in which a stable closed state is not necessary for function\textsuperscript{33}. This proposal, however, is in conflict with the observation that Grp94 is an ATPase\textsuperscript{35}, and like other Hsp90 family members the biological function of Grp94 is dependent on ATP hydrolysis\textsuperscript{36}. It is noteworthy, however, that Grp94 has a very low rate of ATP hydrolysis compared to other Hsp90 family members. This slow ATPase implies a slow rate of closure with AMPPNP, and therefore suggests that the previous structural work on Grp94 may have been performed out of equilibrium.
Results

The very slow rate of Grp94 ATP hydrolysis is illustrated in Figure 2A. The ATPase activity of Grp94 at different temperatures was measured using a previously reported coupled enzymatic assay\textsuperscript{31} (Methods). At room temperature the activity is barely measureable, whereas, increasing temperature dramatically enhances the ATP hydrolysis rate. The rate enhancement is solely due to Grp94, as the activity can be entirely removed with the Hsp90 inhibitor radicicol. Using the room temperature ATPase rate (0.02 min\textsuperscript{-1}) for the closure of Grp94, the amount of time required to accumulate 50% of the potentially closed population of Grp94 was estimated to be approximately 70 minutes (t\textsubscript{1/2}). Although no information was included about the AMPPNP incubation time in previous studies of Grp94\textsuperscript{33,34}, it is very likely these experiments were not performed with sufficient incubation time to reach the conformational equilibrium.

We thus took advantage of the strong temperature sensitivity of Grp94 activity, for designing a new strategy to trap Grp94 in a closed state (Fig. 2B). Increasing the temperature should increase the rate of Grp94 closure, allowing a complete population shift to the closed state. Subsequently decreasing the temperature will recover the energy barrier and maintain the population of Grp94 in the closed state.
**Figure 2.** The temperature sensitivity of Grp94 ATPase activity suggests a strategy to trap a stable closed state. (A) Temperature dependent increases in Grp94 ATPase activity are fully abolished by a known inhibitor (radicicol: "R"). Buffer conditions: 25 mM TRIS pH 7.5, 150 mM KCl, 200 μM ATP, 200 μM MgCl₂, 1 mM CaCl₂, 1 mM BME. Error bars are the standard error of the mean for at least three measurements. (B) Kinetic trapping model of Grp94. Incubating Grp94 with AMPPNP at high temperature drops the energy barrier and allows the equilibrium to shift towards the closed state. Restoring room temperature conditions after heating traps Grp94 in the closed state.

**Grp94 SAXS analysis reveals a symmetric closed conformation**

To test the feasibility of this kinetic trapping strategy, we collected SAXS data of Grp94 in the presence and absence of AMPPNP after a high-temperature incubation. The raw SAXS scattering data (Fig. 3A) shows comparable scattering intensities at low scattering angles (corresponding to small s values), as expected for proteins at the same concentration. I(s) smoothly approaches a plateau at low s-values, indicating minimal (if any) aggregation. The
scattering at intermediate s-values is different for the apo and AMPPNP samples, suggesting a difference in Grp94 conformation.

The raw I(s) data was converted to a probability distribution function, P(r), by Fourier transform using the Primus program\(^3\) (Fig. 3B, Methods). The P(r) distribution represents the summary of all the pairwise interatomic scattering distances within the dimer. Grp94 with AMPPNP shows a compaction relative to the apo state, which is evident from a shift toward shorter scattering distances. Similarly, Grp94 with AMPPNP shows a compaction relative to an ADP incubated sample (Fig. 3C).

**Figure 3.** Grp94 adopts a stable closed state after high temperature incubation with AMPPNP. (A) The scattering curves for Grp94 with AMPPNP (red) and apo (blue) are visibly different, which indicates a difference in conformation. (B) The P(r) representation of Grp94 scattering indicates an AMPPNP-induced compaction, as indicated by a shift towards shorter interatomic scattering distances. (C) ADP-incubated Grp94 adopts a similar conformation as apo Grp94. Only AMPPNP can trap Grp94 in a stable closed state. Buffer conditions: 25 mM TRIS pH 7.5, 150 mM KCl, 2 mM AMPPNP or 2 mM ADP, 2 mM MgCl\(_2\), 5 mM BME.

While the P(r) analysis suggests a general compaction of Grp94 with AMPPNP, it does not immediately reveal whether any specific compact conformation is being populated and it does not determine whether the scattering data can be described by a single structural state. To address these questions, we compared our experimental SAXS data of the AMPPNP-
incubated Grp94 with calculated SAXS data of modeled closed states with symmetric and asymmetric structures (Methods). Figure 4A shows that the symmetric closed state provides a far better fit to our experimental P(r) curve for Grp94 with AMPPNP. The large deviation between the asymmetric model and our experimental data is shown by a residual analysis (Fig. 4B). We conclude that Grp94 can adopt a uniform closed conformation when allowed sufficient time to close. We hypothesize that the closed state of Grp94 is symmetric. We speculate that the published structure of Grp94 with AMPPNP was captured in an artificially open state due to lack of equilibration time with AMPPNP.

Given that our SAXS data is in conflict with the published crystal structure of Grp94 with AMPPNP, we compared our AMPPNP-incubated SAXS data with the predicted SAXS data from the crystal structure (2O1U). The symmetric closed state (Fig. 4A) provides a much better fit than the open state structure (Fig. 4C). Electron microscopy images also confirm a majority of closed conformation of Grp94 with AMPPNP (data from B. Huang, Fig. S1A), in contrast to previous EM analysis which did not include a high temperature incubation34 (Fig. S1B).

**Figure 4.** SAXS data suggests Grp94 adopts a symmetric closed conformation. (A) The symmetric and asymmetric structures of Grp94 are modeled from 2CG9 (Hsp82: green) and 4IPE (Trap1: purple) using Phyre 238. These modeled structures were used to make predicted SAXS curves using FoXS 39,40 and Primus software37. (B) Grp94 SAXS residual analysis shows that the symmetric model has a better fit to the experimental data versus the asymmetric model. (C) The difference between our experimental data (red) and calculated SAXS data from the 2O1U crystal structure (black) suggests the 2O1U structure is not a good representation of our closed Grp94 conformation.
Initial crystallography screening of AMPPNP-incubated Grp94 yielded several crystals (Fig. S2). However, in routine HPLC screening of our nucleotides, we discovered contaminating AMPPN in our stocks of AMPPNP, with increasing contamination observed at high temperature (Fig. 5A). Simple numerical simulations show that small quantities of contaminating AMPPN could lead to large heterogeneity in the nucleotide state of the Grp94 dimer (Fig. 5B), which could severely hamper resolution for crystallography. Therefore, we sought methods to rationally stabilize the closed conformation of Grp94, with the goal of a construct that can close at lower temperature.

**Figure 5.** AMPPNP spontaneously hydrolyzes to an ADP-like AMPPN during pre-incubation at 40° C in the absence of Grp94. (A) C18 reverse phase chromatography can separate AMPPNP from AMPPN and reveals that high temperature incubation increases the AMPPN population from 6% to 9% as calculated from the peak area. (B) Numerical simulations show that the population of AMPPN could dramatically affect the population of Grp94 heterodimers in which one arm contains an AMPPNP molecule and the other contains an AMPPN molecule. The post-incubation AMPPN population from panel A is labeled with a dashed line. The resulting population of Grp94 heterodimers is generated based on the assumption that different nucleotides bind to Grp94 with the same affinity.
Rationally engineering Grp94 activating mutations

With the goal of developing a Grp94 construct with a more stable closed state, we tested two mechanistically unique strategies: (i) Mutations that stabilize the closed state relative to the open state; (ii) Mutations that destabilize the open state relative to the closed state. We designed mutations to test these two strategies and measured their ATPase activity. As discussed below, many of these mutations are strongly activated, indicating both strategies are viable options. By using the closed state model of Grp94 to design these mutations, we also critically tested whether our SAXS derived structural model can provide information that is relevant to Grp94’s ATPase activity.

Method 1: Stabilizing the Grp94 closed state relative to the open state

Because Hsp90 undergoes dramatic conformational changes in the open to closed transition, we reasoned that it may be possible to introduce mutations that reduce the hydrophobic exposed surface area more in the closed state than in the open state. These mutations are expected to preferentially stabilize the closed state. The degree to which the mutated residue is exposed in the transition state will determine the degree to which the mutation will affect closure rate, and therefore activity.
Figure 6. Grp94 activation by close state stabilization. (A) Solvent accessible surface area (SASA) analysis on Grp94 identifies residues that are more exposed in the closed state relative to the open state. (B) ATPase saturation curves show that mutating these exposed hydrophobic residues to alanine increases Grp94 activity. Radicicol controls (square) show no contaminating ATPase. Buffer conditions: 25 mM TRIS pH 7.5, 150 mM KCl, MgCl$_2$ concentration is matched to the ATP concentration, 1 mM CaCl$_2$, 1 mM BME, 40° C. Error bars are the standard error of the mean for at least three measurements.

To identify mutagenesis candidates, we conducted a surface area analysis using GetArea$^{41}$ to identify regions of Grp94 with large changes in hydrophobic SASA between the open and closed states. We calculated fractional changes, $f$, in SASA for hydrophobic side chains normalized to their fully exposed values. Large values of $f_{\text{closed}} - f_{\text{open}}$ indicate residues that are more exposed in the closed versus open state. Several candidates emerged from our analysis: L120, L175, M178, M336, L444, and V446 (Fig. 6A). L241 (Fig. 6A, asterisk) also shows a significant increase in calculated SASA, although this change can largely be attributed to a single neighboring side chain configuration, therefore we did not consider this site.

The L120A, L175A, and M178A mutations are highly activating (Fig. 6B). ATPase saturation curves fit with the Michaelis Menten equation show 2-8 fold increases in $k_{\text{cat}}$ relative to wild-type Grp94. Although $K_m$ values change between 10-50 μM, it is not yet clear how to interpret these changes. We conclude that reducing hydrophobic surface area in the closed state is a viable approach for rationally increasing Grp94 activity. We further conclude that our
closed state model structure is relevant to Grp94’s ATPase activity. Future experiments are needed to determine the degree to which these mutants specifically stabilize the closed state relative to the open state. We plan to construct L444A and V446A, as these sites are in contact with L120, and therefore these mutations are expected to activate Grp94.

Method 2: Destabilizing the Grp94 open state relative to the closed state

We stabilized the closed state by analyzing the modeled closed structure. To complement this approach, we tested whether destabilizing the open state could achieve the same ends. However, it is challenging to introduce hydrophobic surfaces into the open structure that would not result in a steric clash if they were buried in the closed state. Therefore, we sought an alternative method to destabilize the open state.

![Figure 7. Grp94 activation by open state destabilization. (A) In the Grp94 open state, a modeled rotamer of K364 is close to the γ-phosphate of AMPPNP. (B) In the Grp94 closed state, K364 is far from the nucleotide. (C) The K364A mutation increases Grp94 ATPase. Solid lines are fits to the Michaelis-Menten equation. Buffer conditions: 25 mM TRIS pH 7.5, 150 mM KCl, 1 mM CaCl₂, MgCl₂ concentration is matched to the ATP concentration, 40° C. Error bars are the standard error of the mean for at least three measurements.]

Recent findings with bacterial Hsp90 has revealed a salt bridge that is formed in the open state between H255 and the bound nucleotide (Yi Jin, unpublished), while the salt bridge cannot be formed in the closed state. Although H255 is unique to bacterial Hsp90, Grp94 has a
lysine at this position, K364. However, in the AMPPNP-bound Grp94 crystal structure (2.4 Å resolution) the K364 side chain is not visible. Nevertheless, modeled rotamers of K364 can result in a close contact with the bound nucleotide. Figure 7A shows that the closest distance between the γ-phosphate of AMPPNP and the ε-NH₂ group of lysine side chain is 4.2 Å. In contrast, this distance in the closed state is increased to 28.0 Å (Fig. 7B). This structural analysis suggests that the K364A mutation may break a stabilizing salt bridge specific to the open state. Indeed, the K364A mutant has a higher ATPase activity (~5 fold) versus the wild type (Fig. 7C). Based on this example of Grp94 activation by open state destabilization, we will identify and test more unique contacts within the open state that can be disrupted.
Discussion

Previous work on Grp94 revealed a surprising open conformation in the presence of AMPPNP\textsuperscript{33}. Although this preference for the Grp94 open conformation was supported by SAXS and EM measurements\textsuperscript{34}, we were concerned that the low rate of Grp94 ATP hydrolysis (Fig. 2A) may indicate a slow approach to equilibrium in these previous studies. Here, we use a high temperature AMPPNP incubation strategy (Figure 2B) to accumulate a uniform closed population of Grp94. SAXS measurements and modeling suggest a symmetric closed conformation for Grp94 (Figures 3, 4). Previous structural comparisons between Hsp82 and Trap1 suggested that the symmetric closed conformation could be only populated by symmetric cochaperone binding\textsuperscript{21}. Our results here show that a symmetric closed conformation of Hsp90 can be intrinsically favorable. Future comparisons between Trap1 and Grp94 may illuminate why different Hsp90 homologs favor symmetric versus asymmetric closed states, and how these conformations are linked to their function.

Although crystallography would seem a natural extension of our findings on Grp94, we were concerned that the high temperature incubation may inadvertently populate heterodimers with AMPPNP/AMPPN on opposite arms (Fig. 5), potentially threatening diffraction resolution. Therefore, we attempted to engineer Grp94 variants that could adopt a closed conformation at low temperatures. We tested strategies to both stabilize the closed state and destabilize the open state. The success of these strategies validates the use of our closed state model structure.

In the strategy of stabilizing the closed state, we identified hydrophobic side chains that are preferentially exposed in the closed configuration (Fig. 6A). Three such mutations
significantly enhanced activity (Fig. 6B). Our results are conceptually similar to a reverse hydrophobic effect performed in the Sauer lab\textsuperscript{42} in which a folded protein, Cro, is stabilized by removing a solvent accessible phenylalanine side chain. However, to our knowledge this approach has not been extended to stabilizing different conformational states of macromolecular machines. The next step with these mutations is to determine the degree to which our activity changes are reflected in closure and reopening rates, possibly by a previously established FRET assays\textsuperscript{22}.

Interestingly, a genetic screen on yeast Hsc82 identified a T22F mutation that results in a 3-fold ATPase activation\textsuperscript{43}. The T22F mutation would be expected to increase the hydrophobicity open state, but be buried in the closed state. Our results provide a framework for rationalizing this activating mutation and suggests that hydrophobic surface area changes are playing a critical role in regulating Hsp90’s ATPase rate. More mutations (M336, L444, and V446) are needed build a quantitative model to explain our activation mutations in terms of their changes of hydrophobic SASA. Furthermore, positive controls, involving mutations that equally change the hydrophobicity of the open and closed states, are also needed to validate our engineering strategy.

Our second strategy for stabilizing the closed state is via destabilizing the open state. We found one such mutation, which we believe involves a salt bridge between K364 and the bound nucleotide that can only be formed in the open state. Our observed activation by the K364A mutant (Fig. 7B) is striking because the K364 side chain is not visible in the crystal structure (Fig. 7A).

In conclusion, our results indicate that Grp94 can adopt a stable symmetric closed state in the presence of AMPPNP. Using our modeled structure of closed Grp94 we have rationally engineered mutations with increased activity with the ultimate goal of making a Grp94 construct that can close at low temperatures for crystallography.
Methods and Materials

The purification of Grp94 is similar to the described method for other Hsp90 proteins. Grp94 was expressed in E. coli strain BL21* at 30 °C in LB culture medium and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were collected using centrifugation and lysed by sonification with 1 mM PMSF and protease inhibitor tablets (Pierce). The supernatant was collected and loaded onto a Histrap column (GE). The proteins were separated using an imidazole gradient from 40 to 500 mM. Eluted protein was then purified by anion exchange chromatography on a MonoQ column (GE) and by gel filtration on a Superdex S-200 column (GE). Proteins were concentrated to 2-20 mg/mL and flash frozen in 5% glycerol. For crystallography constructs, the poly-histidine tag was cleaved using TEV protease before the anion exchange chromatography.

ATPase activity assay

Enzyme-linked ATPase activity measurements were performed on a temperature-controlled plate reader (BioTek). Briefly, ATP hydrolysis rates were calculated by the consumption of NADH as measured by absorption at 340 nm. Background measurements without chaperone protein were measured prior to adding the chaperone to the plate. After adding the chaperone, a 5-minute shaking step ensured complete mixing. The radicicol control experiments were performed with 10 μM radicicol.

SAXS measurement and data analysis

Grp94 was incubated with 2mM AMPPNP or ADP at 40°C for 3 hours. Apo Grp94 was not treated at high temperature. Grp94 samples were subjected to high speed centrifugation
prior to being sent out for analysis. SAXS data was collected by mail-in delivery at the SIBYLS Beamline at the ALS. Data was taken with three integration times (0.5, 1, and 6s). The 1s integration was used for all analysis.

The symmetric and asymmetric modeled structures of Grp94 were built using Phyre2\textsuperscript{38}. Calculated SAXS $I(s)$ values for the symmetric, asymmetric and 2O1U structures were generated using FoXs software\textsuperscript{39,40} and then converted to a $P(r)$ function via Primus\textsuperscript{37}.

**AMPPNP and AMPPN HPLC separation**

AMPPNP was separated from AMPPN using a C18-AR reverse phase column (ACE). The nucleotide samples were diluted in the 0.1 M KH$_2$PO$_4$ at pH 8.0 before loading onto the column. The nucleotides were separated with isocratic flow with 0.1 M KH$_2$PO$_4$ at pH 6.0. The data was exported using Chem station (Agilent Technologies) and the amount of nucleotides were calculated based on the ratio of the peak area and the total nucleotide concentration.

**Mutagenesis**

Grp94 mutagenesis were conducted using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Briefly, the oligonucleotide primers were ordered from Integrated DNA Technologies and mixed with the solutions provided by the mutagenesis kit according to the protocol. After the thermal cycling in the 2720 Thermal Cycler (Applied Biosystems), the template plasmid was removed by DpnI, while the recombinant plasmid was transformed into TOP10 cells. The TOP10 cells were grown at 37°C overnight before the plasmid extraction using QIAprep Spin Miniprep Kit (Qiagen). Finally the replicated plasmid was transformed in BL21* for further purification.
References


Supplemental Figures

**Supplemental Figure 1.** Electron microscope image confirms individual closed Grp94 molecules post-incubation. (A) A majority of closed Grp94 dimer has been observed from a recent EM image taken by Bin Huang. Arrows mark the open Grp94 molecules. (B) Previously published data from Krukenberg et al. shows an EM image of the open Grp94 molecules in the presence of AMPPNP without incubation (cite). Arrows mark the closed Grp94 molecules.
**Supplemental Figure 2.** Grp94 crystal from crystallography screening. Buffer conditions: 0.1 M BIS-TRIS pH 6.5, 0.2 M LiSO₄, 25% PEG 3350.