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Hsp90 sensitivity to ADP reveals hidden regulation mechanisms

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Abstract

The ATPase cycle of the Hsp90 molecular chaperone is essential for maintaining the stability of numerous client proteins. Extensive analysis has focused on ATP-driven conformational changes of Hsp90, however, little is known about how Hsp90 operates under physiological nucleotide conditions in which both ATP and ADP are present. By quantifying Hsp90 activity under mixed nucleotide conditions we find dramatic differences in ADP-sensitivity among Hsp90 homologs. ADP acts as a strong ATPase inhibitor of cytosol-specific Hsp90 homologs, whereas organellar Hsp90 homologs (Grp94 and TRAP1) are relatively insensitive to the presence of ADP. These results imply that an ATP/ADP heterodimer of cytosolic Hsp90 is the predominant active state under physiological nucleotide conditions. ADP-inhibition of human and yeast cytosolic Hsp90 can be relieved by the cochaperone aha1. ADP-inhibition of bacterial Hsp90 can be relieved by bacterial Hsp70 and an activating client protein. These results suggest that altering ADP-inhibition may be a mechanism of Hsp90 regulation. To determine the molecular origin of ADP-inhibition, we identify residues that preferentially stabilize either ATP or ADP. Mutations at these sites can both increase and decrease ADP-inhibition. An accounting of ADP is critically important for designing and interpreting experiments with Hsp90. For example, contaminating ADP is a confounding factor in FRET experiments measuring arm closure rates of Hsp90. Our observations suggest that ADP at physiological levels is important to Hsp90 structure, activity, and regulation.

Keywords

Hsp90; chaperone; inhibition; regulation; ATPase

Introduction

ATP-dependent heat shock proteins such as Hsp60, Hsp70, Hsp90 and Hsp104, function as molecular chaperones by using ATP binding and hydrolysis to cycle between nucleotide-specific conformations to promote proper folding of a select group of “client proteins” [1, 2]. For Hsp90, ATP-competitive inhibitors cause these clients to become degraded. Longstanding effort has been devoted to uncovering the structural and kinetic details of the

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Hsp90 ATP hydrolysis cycle, with the ultimate goal of understanding how ATP-driven conformational changes of Hsp90 are coupled to client folding and maturation.

The metazoan Hsp90 family includes members exclusive to the cytosol (Hsp90 α and Hsp90 β), endoplasmic reticulum (Grp94) and mitochondria (TRAP1). Yeast and bacteria solely express cytosolic Hsp90 (Hsp82/Hsc82 and HtpG, respectively). Cytosolic Hsp90 homologs are heavily regulated by cochaperones. For example, the cochaperone Aha1 activates Hsp90 [3, 4], whereas Hop and p23 inhibit the ATPase [5, 6]. Some client proteins have also been found to activate Hsp90, for example the ribosomal subunit protein L2 activates HtpG [7]. HtpG can interact directly with DnaK (a bacterial Hsp70 homolog), resulting in enhanced activation from L2 [8]. The organellar Hsp90 homologs have far fewer reported cochaperones. It has not been explained why cytosolic Hsp90 homologs are more cochaperone regulated than Grp94 and Trap1.

Extensive structural analysis of diverse Hsp90 homologs (including crystallography, SAXS, FRET and electron microscopy) has led to a working model of the Hsp90 ATPase cycle [9–23]. Under apo and ADP conditions the Hsp90 dimer adopts a conformationally heterogeneous, and catalytically inactive, ensemble of open configurations in which the N-terminal domains (NTDs) of each monomer are predominantly separated. ATP binding at the NTD stabilizes a transiently populated closed conformation, allowing for ATP hydrolysis and subsequent chaperone reopening. Symmetric and asymmetric closed conformations have been observed for Hsp82 and TRAP1, where the predominant asymmetry occurs at the interface between the middle domain (MD) and C-terminal domain (CTD) [13, 15]. These structures have prompted a two-step conformation proposal in which the closed ATP/ATP homodimer changes its conformation after one ATP has been hydrolyzed, resulting in a structurally distinct ATP/ADP heterodimer [15].

The two-step conformation proposal demands that Hsp90 is in an ATP/ATP state prior to closure, which invites scrutiny of the ATP and ADP nucleotide occupancy of Hsp90 under physiological nucleotide concentrations. Indeed, previous biochemistry suggests that different Hsp90 homologs may populate ATP/ATP and ATP/ADP nucleotide states at different levels. Specifically, binding measurements suggest cytosolic Hsp90s exhibit higher affinity for ADP than ATP [24–26], implying that a high proportion of the ATP/ADP heterodimer state is expected under mixed nucleotide conditions. In contrast, the organellar Hsp90 homologs, Grp94 and TRAP1, have comparable binding affinities for ADP and ATP [27, 28]. However, previous nucleotide binding measurements have been conducted with a single nucleotide species and often using isolated domains of Hsp90. As a result, little is known about how Hsp90 functions under physiological mixtures of ATP/ADP under turnover conditions where the chaperone is undergoing its nucleotide driven conformational cycle.

Indeed, despite substantial progress in understanding the structural role of ATP for Hsp90, relatively little is known about the structural role of ADP. Conformational specificity of Hsp90 for ADP and ATP has been proposed from single molecule experiments [19]. A transiently populated compact ADP-bound conformation of Hsp90 has also been proposed [29], but functional consequences of this proposed state have yet to be observed.

Nevertheless, it is clear that ADP plays a critical role for many ATP-dependent heat shock proteins. For Hsp70, hydrolysis of ATP to ADP induces a dramatic conformational change that traps the substrate protein [2]. For Hsp104, ADP strongly represses ATPase activity, and interestingly, this ADP inhibition is relieved by interactions with Hsp70 [30]. Here we investigate the structural and functional consequences of ADP for the Hsp90 family of chaperones.

Results

We first sought a method for determining the effect of ADP on Hsp90 activity. Hydrolysis rates are typically measured with an enzyme-linked ATP regenerating system, which precludes controlling the ADP concentration. Therefore we utilized an alternative HPLC-based approach to measure hydrolysis rates with a precise quantitation of ADP levels (Methods). Control experiments with known quantities of nucleotide show this method is sensitive and accurate (Figure S1A). Figure 1A shows a chromatographic separation of ATP and ADP from an Hsp82 ATP hydrolysis reaction quenched at hour intervals. Decreased ATPase with increased ADP is evident by the greater production of ADP in the first hour (red line vs black line) versus the second hour (black line vs. blue line). ADP production is linear with time only at short time points (Figure 1B inset), suggesting strong ADP inhibition. A polynomial fit to the data in Figure 1B indeed shows a precipitous loss of activity from only modest levels of accumulated ADP (Figure S1B).

To ensure that the observed ADP-inhibition is not due to nonspecific inactivation during the long measurement periods and to obtain more accurate hydrolysis rates from linear fits, separate experiments were performed at defined ADP levels. Figure 1C shows an example of multiple Hsp82 hydrolysis experiments, each performed at different ADP fractions ranging from 2–90%. ATPase rates are calculated from the slopes in Figure 1C (equation 3), which indeed show strong ADP inhibition (Figure 1D). This ADP inhibition assay gives highly reproducible results (Figure S2A). An Hsp90-specific inhibitor, NVP-AUY922, abolishes ATP hydrolysis indicating the absence of contaminating ATPases ('x' symbols, Figure S2A).

Hsp82 activity measured with an ATP-regenerating assay ($4.95 \pm 0.12 \text{ min}^{-1}$, Figure 1D, green diamond) differs by 20% from the first HPLC ATPase measurement (3.9 min^{-1}). However, the first HPLC measurement has ~2% ADP due to the requirement of accumulated ADP to measure the rate. The strong ADP inhibition of Hsp82 suggests that 2% ADP may explain this 20% rate discrepancy. To test this idea, we developed a curve-fitting quantification method based on a competitive inhibition model (equation 4, Methods). Traditional competitive inhibition experiments with variable ADP and fixed ATP concentrations confirm that this model is appropriate (Figure S2B).

Non-linear least squares fitting of the ADP inhibition model to the HPLC ATPase data (dashed lines, Figure 1D) yields two parameters, one of which is the activity in the absence of ADP (5.1 ATP/min), similar to the ATPase measured under ATP-regenerating conditions. The second fit parameter is the ratio of the ATP and ADP apparent binding affinities, referred to as the ADP-sensitivity factor (R), which quantifies the degree of ADP inhibition. This quantification shows that under turnover conditions Hsp82 favors ADP over ATP by a

factor of 17. Replicate experiments show small error on the ADP sensitivity factor ($R = 16 \pm 0.7$, Figure S2A). Temperature and pH strongly influence Hsp82 ADP inhibition (Figure S3A, B), whereas salt has a minimal consequence (Figure S3C).

Figure 2 shows that highly divergent cytosolic Hsp90 homologs HtpG and Hsp90 α exhibit strong ADP inhibition, with Hsp90 α showing the highest sensitivity ($R = 66$). In contrast, the organelle-specific Hsp90 homologs Grp94 and TRAP1 are relatively insensitive to ADP ($R = 0.4$ and 0.6 respectively, Figure 2C, D). These diverse Hsp90 homologs with very different levels of ADP-inhibition can all be adequately described by the ADP inhibition model (dashed lines).

We used the ADP inhibition model to predict populations of nucleotide-bound dimer species at different ADP levels (Supplementary Information, equations 11–15). Figure 2E shows predicted nucleotide states for human cytosolic Hsp90 α ($R = 66$). The ATP/ATP homodimer population drops dramatically in the presence of ADP making the ATP/ADP heterodimer the predominant active species by ~1.5% ADP. By ~3% ADP, the majority of Hsp90 α is in an inactive ADP/ADP state. We conclude that at physiological ADP levels (5–15% [31]) the dominant active species of cytosolic Hsp90 is an ATP/ADP heterodimer. In contrast, the predicted nucleotide states of Grp94 ($R = 0.4$, Figure 2F) show that the ATP/ATP homodimer of Grp94 is the dominant species over a large range of ADP levels.

Regulation of ADP inhibition by cochaperones and client proteins

The dramatic impact of ADP on cytosolic Hsp90 suggests a potential source of regulation. To test this idea, we measured Hsp82 ADP-inhibition in the presence of a highly activating cochaperone, Aha1 [3, 4]. Figure 3A and 3C show that Aha1 enhances activity and relieves ADP inhibition, reducing the R value by a factor of two. Similar results were observed for Hsp90 α in the presence of human Aha1 (Figure S4). We also observe a similar reduction of HtpG ADP inhibition by an activating client protein, the ribosomal protein L2 (Figure 3B, C). Our findings suggest that ADP inhibition provides a regulatory mechanism to restrict ATP consumption when the chaperone is not interacting with activating cochaperones and client proteins.

Recent findings with HtpG suggest a direct interaction with the Hsp70 homolog, DnaK, and synergistic ATPase activation of HtpG/L2/DnaK [8]. In agreement with Genest et al. we observe greatly enhanced activity of HtpG/L2/DnaK, as well as a further reduction of ADP inhibition (Figure 3B, C). DnaK activity is negligible in comparison with HtpG in these experiments (inset, Figure 3B). We conclude that excluding physiologically-relevant levels of ADP in experiments with Hsp90 can mask the full activating power of a cochaperone and client protein. This point is illustrated in Figure 3D, which shows the increased fold activity of HtpG by DnaK and L2 at increasing levels of ADP, revealing a much larger fold activation at physiological nucleotide concentrations [31] than under ATP-regenerating conditions. Interestingly, addition of DnaK to HtpG relieves ADP inhibition to a similar extent as L2 (Figure 3B, C), despite DnaK having a minimal impact on activity in the absence of ADP. This suggests a unique mechanism by which DnaK regulates HtpG ADP-inhibition.

The Hsp90 closed state is minimally sensitive to ADP

ADP inhibition of cytosolic Hsp90 implies that ADP outcompetes ATP under ATP turnover conditions, which is an important consideration in the design and interpretation of experiments measuring Hsp90 ATPase activity. We then sought to determine the effect of ADP on the conformation of Hsp90 in equilibrium experiments. To address this question we utilized small angle x-ray scattering (SAXS) to measure the Hsp90 open/closed conformational equilibrium with ADP and a non-hydrolyzable ATP (AMPPNP), to ensure the system is at equilibrium.

The SAXS $P(r)$ curve is a convenient visualization of the open/closed conformational equilibrium, whereby the open state has significant scattering beyond 130 Å and the closed state does not. We selected pH 9.0 for these experiments as HtpG exhibits two-state behavior (open/closed) under these conditions, whereas at lower pH values HtpG is better described by three conformational states [11]. Indeed in all the following SAXS measurements (Figure 4A and 4C) the family of $P(r)$ curves show an isosbestic point at 75 Å, suggesting that only the open and closed states are present. The radius of gyration calculated from Guinier analysis quantifies the changing open/closed state population (Figure 4B and 4D).

We first tested HtpG with varying concentrations of AMPPNP alone. The extent of closed state accumulation is thermodynamically linked to the closed state affinity for AMPPNP. Under our conditions AMPPNP results in full closure [11], therefore we expect that AMPPNP should bind favorably to the closed state. Indeed, Figure 4A and 4B show a very high apparent closed state affinity for AMPPNP ($K_{d,app}$ of 12–15 μM, Methods). This affinity is much greater than the apparent affinity for ATP under turnover conditions (K_m of 1200 μM, inset). These results suggest that the ATPase K_m reflects weaker ATP binding properties of the open state. Indeed, FRET and SAXS measurements show that under ATP turnover conditions HtpG primarily populates an open conformation (Figure 6A and Figure S5). The large K_m value of HtpG is a result of a pH-dependent open-state nucleotide binding mechanism, as discussed elsewhere [32].

We next tested the closed state stability in the presence of mixtures of ADP and AMPPNP. Given the high apparent affinity of AMPPNP for the closed state, we anticipate that the Hsp90 closed state accumulation should be relatively insensitive to ADP. Indeed, no significant change in open/closed equilibrium is observed up to ~30% ADP (Figure 4C and 4D) from two comparable datasets (red and blue symbols). Despite a modest level of variation, both datasets yield an apparent ADP-sensitivity factor of the closed state ($R_{app}=0.4$) that is much lower than the ADP-sensitivity factor measured under ATP turnover conditions ($R=15$, Figure 2A). We conclude that the closed state accumulation of HtpG is minimally sensitive to ADP.

Residues contributing to ADP inhibition

To gain structural insight into the origin of ADP inhibition we sought residues that make stabilizing contacts specifically to either ATP or ADP. Inspection of an ADP-bound Hsp82 NTD crystal structure shows K98 making contact to the ADP β-phosphate (Figure 5B).

Since terminal nucleotide phosphate groups carry a greater negative charge than the preceding group, an electrostatic interaction between K98:β-phosphate of ADP will be stronger than the interaction between K98:β-phosphate of ATP. Removing K98 is therefore expected to equalize ADP/ATP binding, and indeed, the K98A variant exhibits less ADP inhibition than wild-type (Figure 5A).

Recent findings suggest that K364 on the Grp94 MD can form a stabilizing electrostatic interaction with ATP [32]. Modelling indicates that K364 can form a stabilizing salt bridge with the ATP γ-phosphate in the open conformation (Figure 5D), but is too far removed to form a salt bridge with the β-phosphate. Therefore, we anticipate that K364 stabilizes ATP over ADP, predicting that a mutation at this site will increase ADP inhibition of Grp94. Figure 5C confirms this expectation. The origin of the increase in activity for K364A is discussed elsewhere [32]. Hsp82 has a polar residue (N298) at the equivalent sequence position (Figure S6A), and since the N298A mutation does not alter ADP-inhibition (Figure S6B) we ascribe the ADP-inhibition influence of K364 on electrostatics. We conclude that nucleotide-specific stabilizing contacts contribute to ADP-inhibition.

ADP-sensitivity is a confounding factor in FRET experiments

Hsp90 arm closure rates measured via FRET with non-hydrolyzable ATP analogs (AMPPNP or ATP-γ-S) are often correlated with ATPase activity [18, 33, 34]. However, the rate of closure is found to be an order of magnitude slower than ATPase [17, 18]. Given the strong effect of ADP on activity we tested whether ADP could play a confounding role in FRET experiments. HPLC analysis of AMPPNP reveals 5% AMPPN in AMPPNP stock solutions (Figure 6B), therefore FRET experiments have a minimum of 5% of an ADP-like product. HPLC analysis of ATP-γ-S stock solutions reveals a contaminating nucleotide species present at quantities of at least 20% (data not shown). FRET experiments measuring arm closure with added ADP (Figure 6A) show that closure is decelerated by ADP. The closure rate dependence on fraction ADP/AMPPN (Figure 6C) follows the strong ADP-inhibition observed in activity experiments (Figure 2A). Adding further complication, an additional 1.5% AMPPNP is converted to AMPPN during the FRET experiment, such that the concentration of this ADP-like contaminant changes throughout the measurement. We can therefore only put limits on the fraction ADP+AMPPN in these closure experiments (Figure 6C, blue and red symbols). We propose that contaminating ADP-like products contribute to the discrepancy between ATP hydrolysis rates and arm closure rates of Hsp90. Arm closure rates are slow compared to measured ADP off rates [20, 25], suggesting that closure is not under kinetic control of ADP release.

To test whether contaminating ADP prevents the accumulation of closed state under ATP conditions, FRET experiments were performed with HtpG in the presence of ATP and the ATP-regenerating components pyruvate kinase and phosphoenolpyruvate (Figure 6A). No closed state accumulation was observed under these conditions, showing that HtpG primarily populates the open state under turnover conditions. Similar results are observed for apo and ADP conditions.

Discussion

By developing an assay and quantification method to determine the effect of ADP on Hsp90 activity we show that cytosolic Hsp90 homologs are very sensitive to ADP whereas the organellar Hsp90 homologs are relatively insensitive. ADP-sensitivity is important to the design and interpretation of biochemical and biophysical experiments on cytosolic Hsp90. For example, the extraordinary inhibition of Hsp90 α (Figure 2B) suggests a source of error for quantifying activity via radiolabeled ATP and phosphate release, due to variable levels of ADP. Similar experimental errors will arise in biochemical experiments performed in lysates or *in-vitro* experiments where Hsp90 is incubated with ATP prior to the measurement. For these cases the duration of incubation and concentration of Hsp90 and other ATPases will affect the structure and activity of Hsp90 continuously during the course of the experiment due to accumulated ADP.

Although an ATP-regenerating system can avoid the difficulties discussed above, excluding ADP from Hsp90 masks interesting behavior. For example, Hsp90 ADP-sensitivity reveals a previously hidden method of regulation. Cochaperones and client proteins can activate Hsp90 by relieving ADP inhibition (Figure 3), a regulatory effect that is invisible under ATP-regenerating conditions. Indeed, DnaK relieves ADP-inhibition with only a modest influence on activity when ADP is absent. This regulatory role of DnaK on HtpG is similar to the effect of Hsp70 on Hsp104 ADP inhibition [30]. More work is needed to uncover the nucleotide regulation mechanism of Hsp70-Hsp90 complexes. Our results are consistent with recent findings that HtpG and DnaK can form a functional complex despite the absence of a Hop-like cochaperone [8]. Cytosolic Hsp90 homologs are heavily regulated by cochaperones and are ADP-inhibited, whereas organellar homologs Grp94 and TRAP1 have fewer cochaperone interactions and are relatively insensitive to ADP (Figure 2). We propose that cochaperones and client proteins play a critical role in alleviating ADP-inhibition. However, we anticipate that different Hsp90 homologs may regulate ADP-inhibition by a wide variety of mechanisms.

Under physiological nucleotide concentrations (5–15% [31]) we predict that cytosolic Hsp90 primarily populates two states that are absent in ATP-regenerating conditions: ADP bound to both arms, and ATP bound to one arm and ADP bound to the opposite arm. Although the ATP/ADP nucleotide heterodimer is expected to be the dominant hydrolytically active species for cytosolic Hsp90 at cellular nucleotide concentrations (Figure 2E), little is known about this asymmetric nucleotide state of Hsp90. Numerous studies suggest that structural asymmetry is a key component of Hsp90 function, including an asymmetric crystal structure, asymmetric cochaperone interactions, and asymmetric *in vivo* functional analysis [15, 35–37]. An asymmetric ADP/ATP nucleotide state of Hsp90 may confer important structural and functional consequences to the dimer.

SAXS results with HtpG show that the closed state population is relatively unaffected by contaminating ADP (Figure 4C and 4D). In contrast, ADP strongly decelerates kinetic transitions from the open state to the closed state (Figure 6). Under our experimental conditions, we find that HtpG primarily adopts an open configuration under ATP turnover

conditions (Figure 6A and Figure S5). This data is inconsistent with conclusions drawn from single molecule experiments on HtpG [21]. More work is needed to clarify this discrepancy.

We identify mutations that both increase and decrease ADP-inhibition (Figure 5). It is likely that the determinants of ADP and ATP affinity are widely distributed with many more contributing groups. The strong temperature dependence of ADP-inhibition (Figure S3A) suggests that conformational flexibility may play a role in the different binding affinities of ADP and ATP. More work is needed to determine the molecular origins underlying Grp94 and TRAP1 insensitivity to ADP relative to cytosolic Hsp90 homologs.

SAXS measurements show that the accumulation of the HtpG closed state is minimally sensitive to ADP (Figure 4). Our interpretation of this data is that the Hsp90 preference for ADP over ATP is conformation specific, whereby the open state favors ADP over ATP whereas the closed state favors ATP over ADP. A linkage between Hsp90 conformation and nucleotide preference provides a plausible mechanism by which L2 and Aha1 reduce ADP inhibition (Figure 3). Specifically, since both L2 and Aha1 activate their respective Hsp90 homologs, they therefore push the conformational equilibrium towards the closed state, whose conformation favors binding ATP over ADP.

Materials and Methods

Protein purification

Purification of HtpG, TRAP1, Hsp90 α , Grp94, Hsp82, and Aha1 has been described previously [4, 9, 15, 29]. In short, proteins were expressed in *E. coli* strain BL21* at 37 °C in LB. Expression was induced via addition of 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). Hsp82, Grp94, and Hsp90 α cultures were incubated at 30 °C following addition of IPTG. Cells were pelleted by centrifugation followed by sonication in the presence of 1 mM PMSF. The soluble fraction was isolated by centrifugation and loaded onto a nickel-nitrilotriacetic acid column. Weak binding proteins were washed off with 25 mM imidazole followed by elution of the His-tagged protein with 500 mM imidazole. The elution was then further purified with anion exchange chromatography (MonoQ, GE Healthcare) followed by size exclusion chromatography (Superdex S-200, GE Healthcare). Proteins were concentrated to between 5–10 mg/ml and flash frozen in 5% glycerol. L2 was purified as described previously [7].

ADP inhibition measurements

ATPase reactions were initiated by addition of 5 μ M dimer Hsp90 to a solution containing various concentrations of ATP and ADP totaling 5 mM nucleotide, which is in excess of measured K_m values [27, 28, 38, 39]. 5 μ M dimer was used in these experiments because at 37 °C Hsp82 activity has a protein concentration dependence that saturates at 5 μ M (data not shown). Time points were quenched at predetermined time intervals by mixing 5 μ L of the ATPase sample with 5 μ L of 0.2M EDTA pH 8.0. Quenching time intervals were selected such that the change in fraction ADP over time was linear. Protein was precipitated with 5 μ L of a 12% TCA solution, followed by centrifugation at 20,000 xG for 1 minute. TCA precipitation and protein removal were performed quickly to minimize acid hydrolysis of

ATP. 10 μL of the supernatant was mixed with 66.7 μL of 0.1M KH_2PO_4 pH 8.0 to neutralize the pH. Each sample was filtered (SpinX spin filter) prior to HPLC analysis.

ATPase reactions for Hsp90 α , Hsp82, Grp94, and TRAP1 were performed in 10 mM MgCl_2 , 50 mM HEPES pH 7.5, 150 mM KCl, and 2% DMSO at 37 °C. Measurements with Grp94 included 1 mM CaCl_2 . ATPase reactions for Hsp82 + 20 μM Aha1 were performed with 11 mM KCl. ATPase reactions for Hsp90 α $\pm$ human Aha1 (hAha1) were performed with 28 mM KCl. HtpG activity measurements were performed with 5 μM HtpG, $\pm$ 10 μM L2, $\pm$ 10 μM DnaK, in 10 mM MgCl_2 , 50 mM Tris pH 9, 150 mM KCl, and 2% DMSO at 25 °C. DnaK activity measurements were performed with 5 μM DnaK, $\pm$ 5 μM L2, in 10 mM MgCl_2 , 50 mM Tris pH 9, 150 mM KCl, and 2% DMSO at 25 °C. Previous studies have shown that DnaK is folded, active, and capable of being activated by peptide substrates at pH 9 [40]. Inhibitor controls for HtpG, Hsp90 α , and Hsp82 were measured in the presence of 200 μM NVP-AUY922. Grp94 and TRAP1 inhibitor controls were measured in the presence of 100 μM radicicol. Negative ATPase values observed at high fraction ADP in Figures 2B and 2C ('x' symbols and open circles) could indicate a minor contaminating ATP synthesizing enzyme.

HPLC separation and analysis of nucleotides

Nucleotide content was analyzed chromatographically using a C18-AR reversed-phase column (ACE, 250 $\times$ 2.1 mm, 5 μM particle size). Nucleotides were separated isocratically with 0.1M KH_2PO_4 at pH 6.0 at a flow rate of 0.4 ml/min. ATP and ADP peak areas were integrated via absorption at 254 nm to calculate the fraction ADP: ADP peak area/(ADP peak area + ATP peak area). For each ATPase reaction, fraction ADP versus time was fit to a straight line to extract a slope

$$\text{Slope} = \frac{d\left(\frac{[ADP]}{[ADP] + [ATP]}\right)}{dt} \quad (1)$$

Since the total nucleotide concentration ($[ATP] + [ADP]$) is constant

$$\frac{d[ADP]}{dt} = \text{Slope} * ([ADP] + [ATP]) \quad (2)$$

Hsp90 activity can be calculated directly from the slope

$$\text{ATPase} = \frac{\text{Slope} * ([ADP] + [ATP])}{[Hsp90]} \quad (3)$$

ADP inhibition of Hsp90 is quantified with an ATP/ADP competitive binding model (derived in Supplementary Information). The ADP-sensitivity factor, R , is obtained via non-linear least squares fitting (Kaleidograph) of ATPase versus fraction ADP

$$ATPase = \frac{A(1-x)}{1+(R-1)x} \quad (4)$$

where x is the fraction ADP and A is the ATPase in the absence of ADP. This fitting assumes a minimal population of apo protein and that the binding of the first nucleotide does not affect the affinity of the second (Supplementary Information). R represents the ratio of the

effective binding affinities of ADP and ATP under turnover conditions ($R = \frac{K_d^{ATP}}{K_d^{ADP}}$).

Traditional competitive inhibition experiments were performed by using the described HPLC method to measure Hsp82 ATPase with variable concentrations of ADP and fixed concentrations of ATP. The inhibition was quantified using a standard competitive inhibition model (Supplementary Information). For each ATP concentration, the resulting ATPase versus ADP concentration was fit via non-linear least squares to the equation,

$$y = B \left(\frac{[ATP]}{K_d^{ATP} + [ATP]} \right) \left(\frac{1}{1 + K^{obs}x} \right) \quad (5)$$

where B is the activity in the absence of ADP, x is the concentration of ADP, y is the ATP hydrolysis rate, and K_d^{ATP} is the ATP dissociation constant. The fit value for K^{obs} was used to determine the ADP concentration at half inhibition ($[ADP]_{1/2}$). The resulting $[ATP]$ versus $[ADP]_{1/2}$ was fit via linear least squares fitting to the equation,

$$[ATP] = R[ADP]_{1/2} - K_d^{ATP} \quad (6)$$

ATP-regenerating assay

ATP hydrolysis rates under ATP-regenerating conditions were measured with a previously described enzyme coupled assay [15]. In brief, pyruvate kinase and lactate dehydrogenase were used to couple Hsp90 ATPase to NADH oxidation and regenerate ATP. Hsp90 ATP hydrolysis rates were determined by monitoring NADH absorption at 340 nm. ATPase measurements of Hsp82 were performed using a temperature controlled spectrophotometer (Agilent) with 5 μ M dimer Hsp82. Buffer conditions were matched with those in Figure 1D: 5 mM ATP, 10 mM MgCl₂, 50 mM HEPES pH 7.5, 150 mM KCl, 2% DMSO, 37 °C. HtpG ATPase data are from a recent study [32]. The measurements were performed on a temperature controlled plate reader (BioTek) with 1.5 μ M dimer HtpG. Buffer conditions

were: 15 mg/ml bovine serum albumin (BSA), 50 mM Tris pH 9.0, 150 mM KCl, 1:1 stoichiometry of ATP:MgCl₂, 25 °C.

FRET measurements

Arm closure rates were measured with a previously established HtpG FRET pair [17, 18]. Cysteine mutants of HtpG, E62C and D341C, were each purified and labeled with Alexa Fluor 647 (acceptor) and Alexa Fluor 555 (donor) respectively. A 5-fold molar excess of Alexa Fluor maleimide dye was incubated with HtpG mutants for three hours at room temperature, followed by quenching with excess β-mercaptoethanol. Unreacted fluorophore was separated from labeled protein via size exclusion chromatography. FRET competent heterodimers were formed by incubating 250 nM acceptor- and donor-labeled monomer for 30 min at 30 °C. Arm closure was initiated by adding nucleotide. Fluorescence was measured using an excitation wavelength of 525 nm and slit width of 2 nm, and an emission slit width of 3 nm. Arm closure kinetics were measured by monitoring donor emission at 563 nm and acceptor emission at 663 nm. The FRET efficiency, (acceptor)/(acceptor + donor), as a function of time was fit to a single exponential. HPLC (described above) was used to measure the fraction of AMPPNP in the AMPPNP stock solutions as well as the fraction AMPPNP accumulated after 1 hour in a typical FRET reaction. FRET experiments were performed in a buffer of 10 mM MgCl₂, 50 mM Tris pH 9, 150 mM KCl and 2% DMSO at 25 °C. Experiments with AMPPNP and ADP were performed with 5 mM total nucleotide. FRET experiments with ATP under ATP regenerating conditions were performed with 5 mM ATP, 20 units/ml pyruvate kinase from rabbit muscle (Sigma), and 0.4 mM phosphoenolpyruvate (PEP). FRET experiments with ATP or ADP were performed with 5 mM nucleotide.

SAXS measurements

HtpG samples were sent to the SIBYLS beamline [41, 42]. For each nucleotide condition, multiple samples were prepared: one or more sample(s) with a given concentration of HtpG, and a matching buffer sample for buffer subtraction. R_g values in Figure 4D are from 8 μM and 15 μM HtpG samples incubated with 4 mM total nucleotide (AMPPNP and ADP). R_g values in Figure 4B are with 8 μM HtpG. P(r) curves and R_g values were calculated using the ATSAS software suite [43]. All P(r) curves are normalized to have a constant area under the curve. Buffer conditions were 10 mM MgCl₂, 50 mM Tris pH 9, 150 mM KCl, 2% DMSO. The data in Figure 4B were fit to a binding model that accounts for the difference between the free and total AMPPNP:

$$y = \frac{(C_p + K_{d,app} + x) - \sqrt{(C_p + K_{d,app} + x)^2 - 4C_p x}}{2} \quad (7)$$

where C_p is the protein concentration, $K_{d,app}$ is the apparent AMPPNP binding affinity, x is the total concentration of AMPPNP in the sample, and y is the population of the closed, AMPPNP bound, state. The curve was fit using protein concentrations of 8 μM or 16 μM with negligible differences in the resulting $K_{d,app}$ values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Hsp90 activity is measured under mixed ADP/ATP nucleotide conditions
- ADP strongly inhibits cytosol-specific Hsp90s but not organelle-specific Hsp90s.
- Relief of ADP-inhibition is a form of Hsp90 regulation by cochaperones and clients.
- An accounting of ADP is important for designing experiments with Hsp90.

Figure 1.

(A) HPLC separation of ATP and ADP from an Hsp82 ATPase reaction at hour intervals. The red line is the initial time point, and the black, blue, green, and purple are consecutive time points at 1 hour intervals. (B) The fraction ADP is linear only over short time intervals (inset). The red dashed line is a fourth order polynomial fit. (C) HPLC-based ATPase measurements for Hsp82 at defined ADP fractions. The linear fit to each time series (dashed lines) shows decreasing activity with increasing ADP. (D) The corresponding color-matched ATPase values from panel (C) indeed exhibit strong ADP-inhibition. Activity measured using an ATP-regenerating assay (green diamond) is consistent with the HPLC-based assay (circles). The dashed line is a non-linear least squares fit with equation 4. Buffer conditions described in Methods.

Figure 2.

Representative ADP inhibition curves of Hsp90 homologs. The cytosolic homologs HtpG (A) at pH 9.0 (red circles) and pH 7.5 (blue diamonds, $R = 30$), and Hsp90 α (B) both exhibit strong ADP-inhibition, whereas organelle-specific Hsp90 homologs Grp94 (C) and Trap1 (D) are relatively insensitive to ADP. The dashed lines are non-linear least squares fits with equation 4. Minimal ATPase is measured in the presence of an Hsp90 inhibitor ('x' symbols). In (B) and (C), inhibitor-subtracted and unsubtracted rates are shown in red diamonds and open circles, respectively. Buffer conditions described in Methods. (E) Predicted populations of Hsp90 α nucleotide states show an ATP/ADP heterodimer as the

dominant active species by ~1.5% ADP. **(F)** The predicted populations of Grp94 nucleotide states show the ATP/ATP homodimer as the dominant species over a large range of ADP levels.

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Figure 3.

(A) Aha1 activates Hsp82 and relieves ADP inhibition. (B) L2 and DnaK decrease HtpG ADP inhibition while increasing ATPase activity. L2 moderately activates DnaK (inset). The representative ADP inhibition curves shown in (A) and (B) are fit via non-linear least squares with equation 4. (C) Averaged ADP sensitivity factors associated with replicate experiments from panels (A) and (B). Error bars are the standard error of the mean from three separate ADP-inhibition curves. Buffer conditions described in Methods. (D) Fold activation of HtpG from DnaK/L2 at increasing ADP fraction, calculated from ADP inhibition data and curve fitting in panel (B).

Figure 4.

(A) SAXS analysis of HtpG (8 μM dimer) shows high affinity of the closed conformation for AMPPNP. (B) Radius of gyration (R_g) data from panel (A) is fit to a binding curve (equation 7) which shows an apparent closed-state AMPPNP affinity 100-fold higher than the K_m (inset, data from [32]). (C) SAXS analysis of HtpG (15 μM dimer) shows closed state accumulation even the presence of a high fraction of ADP (total nucleotide of 4 mM). (D) R_g values from panel (C) illustrate that the accumulation of closed state is minimally sensitive to ADP. The dashed lines are a non-linear least squares fit with equation 4. Buffer conditions described in Methods.

Figure 5.

(A) K98 contributes to Hsp82 ADP-inhibition. (B) An Hsp82 NTD structure (1AM1) shows a salt bridge between K98 and the β -phosphate of ADP. (C) K364 contributes to the lack of Grp94 ADP-inhibition. (D) Modeled rotamers of K364 in the Grp94 open state (201U) show that a salt bridge can be formed to the ATP γ -phosphate but not the β -phosphate. Dashed lines are a non-linear least squares fit with equation 4. Buffer conditions described in Methods. ADP sensitivity factor error bars are the standard error of the mean from three separate ADP inhibition curves.

Figure 6.

(A) Arm closure rates measured by FRET are decelerated by ADP (red: no added ADP, blue: 5% ADP, green: 10% ADP, purple: 20% ADP). Solid lines are single exponential fits to the data. No accumulation of closed state is observed in the following conditions: ADP, Apo, ATP, and ATP with an ATP regenerating system. (B) HPLC analysis of AMPPNP stock solution shows a 5% AMPPN contamination. (C) Closure rates decrease with increasing fraction ADP+AMPPN. The fraction ADP+AMPPN was determined from the AMPPN present in AMPPNP stock solution (5%, red circles) and the AMPPN accumulated after the

FRET measurement (6.5%, blue diamonds). The dashed lines are a non-linear least squares fit with equation 4. Buffer conditions described in Methods.

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Figure 7.