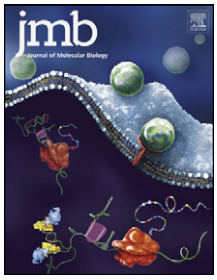

Hsp90 Sensitivity to ADP Reveals Hidden Regulation Mechanisms

Jackson C. Halpin and Timothy O. Street

Department of Biochemistry Brandeis University, *Waltham, MA 02453, USA*

Correspondence to Timothy O. Street: *tstreet@brandeis.edu*
http://dx.doi.org/10.1016/j.jmb.2017.08.005
Edited by J. Buchner

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 organellular 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 fluorescence resonance energy transfer experiments measuring arm closure rates of Hsp90. Our observations suggest that ADP at physiological levels is important to Hsp90 structure, activity, and regulation.

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. Long-standing effort has been devoted to uncovering the structural and kinetic details of the 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 (Hsp90α and Hsp90β), 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 organellular 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, small-angle X-ray scattering (SAXS), fluorescence resonance energy transfer (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 and C-terminal domain [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 that cytosolic Hsp90s exhibit higher affinity for ADP than for ATP [24–26], implying that a high proportion of the ATP/ADP heterodimer state is expected under mixed nucleotide conditions. In contrast, the organellular 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 (Materials and Methods). Control experiments with known quantities of nucleotide show this method is sensitive and accurate (Fig. 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 *versus* black line) *versus* the second hour (black line *versus* blue line). ADP production is linear with time only at short time points (Fig. 1b inset), suggesting strong ADP inhibition. A polynomial fit to the data in Fig. 1b indeed shows a precipitous loss of activity from only modest levels of accumulated ADP (Fig. 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% to 90%. ATPase rates are calculated from the slopes in Fig. 1c (Eq. (3)), which indeed show strong ADP inhibition (Fig. 1d). This ADP inhibition assay gives highly reproducible results (Fig. S2A). An Hsp90-specific inhibitor, NVP-AUY922, abolishes ATP hydrolysis indicating the absence of contaminating ATPases ('x' symbols, Fig. S2A).

Hsp82 activity measured with an ATP-regenerating assay ($4.95 \pm 0.12\ \text{min}^{-1}$, Fig. 1d, green diamond) differs by 20% from the first HPLC ATPase measurement ($3.9\ \text{min}^{-1}$). 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 (Eq. (4), Materials and Methods). Traditional competitive inhibition experiments with variable ADP and fixed ATP concentrations confirm that this model is appropriate (Fig. S2B).

Non-linear least squares fitting of the ADP inhibition model to the HPLC ATPase data (dashed lines, Fig. 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 ($R = 16 \pm 0.7$, Fig. S2A). Temperature and pH strongly influence Hsp82 ADP