Crowding Activates Heat Shock Protein 90*

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Hsp90 is a dimeric ATP-dependent chaperone involved in the folding, maturation, and activation of diverse target proteins. Extensive in vitro structural analysis has led to a working model of Hsp90’s ATP-driven conformational cycle. An implicit assumption is that dilute experimental conditions do not significantly perturb Hsp90 structure and function. However, Hsp90 undergoes a dramatic open/closed conformational change, which raises the possibility that this assumption may not be valid for this chaperone. Indeed, here we show that the ATPase activity of Hsp90 is highly sensitive to molecular crowding, whereas the ATPase activities of Hsp60 and Hsp70 chaperones are insensitive to crowding conditions. Polymer crowders activate Hsp90 in a non-saturable manner, with increasing efficacy at increasing concentration. Crowders exhibit a non-linear relationship between their radius of gyration and the extent to which they activate Hsp90. This experimental relationship can be qualitatively recapitulated with simple structure-based volume calculations comparing open/closed configurations of Hsp90. Thermodynamic analysis indicates that crowding activation of Hsp90 is entropically driven, which is consistent with a model in which excluded volume provides a driving force that favors the closed active state of Hsp90. Multiple Hsp90 homologs are activated by crowders, with the endoplasmic reticulum-specific Hsp90, Grp94, exhibiting the highest sensitivity. Finally, we find that crowding activation works by a different mechanism than co-chaperone activation and that these mechanisms are independent. We hypothesize that Hsp90 has a higher intrinsic activity in the cell than in vitro.

Molecular chaperones play a central role in maintaining folded and active proteins in the cell. Hsp70 class chaperones inhibit misfolding by binding and releasing short hydrophobic segments of unstructured polypeptides with cycles of ATP hydrolysis. Hsp60 class chaperonins promote folding by isolating single protein chains within an isolated cavity. Similar to Hsp70 and Hsp60, Hsp90 has an essential ATPase activity, but the underlying functional mechanism appears to be different. Hsp90 plays important regulatory roles under non-stress conditions by its interactions with specific classes of substrates (“clients”) such as kinases and nuclear receptors (1). Hsp90 has a dimeric structure that can undergo dramatic rearrangements upon ATP binding and hydrolysis. Despite significant progress in characterizing Hsp90’s ATP-dependent conformational cycle in vitro, it is still unclear how Hsp90 performs its many critical cellular functions.

Hsp90 conformational heterogeneity results from rigid-body rearrangements of the three domains within the monomer. The N-terminal domain (site of nucleotide binding) can rotate relative to the middle domain. The middle domain can be positioned against the C-terminal domain (site of dimerization) in multiple orientations, resulting in a flexible and structurally heterogeneous open conformation. Indeed, all full-length Hsp90 structures determined to date have different domain arrangements (2–5) (2CG9, 2IOQ, 2IOP, 2O1U, 4IPE), and solution structures determined by small angle x-ray scattering show even greater conformational heterogeneity (6–9). The resulting diversity of Hsp90 configurations presents a challenge in determining which structures are most biologically relevant and how these configurations are coupled to client folding and activation.

Hsp90s70 exhibit weak activity in vitro, with ATPase rates on the order of 0.1–1 hydrolysis events per minute (10). FRET measurements have shown that arm closure is rate-limiting for ATP hydrolysis (11, 12). The slow rate of closure and rapid subsequent ATP hydrolysis result in Hsp90 predominantly populating an open configuration in the presence of ATP. As a result, non-hydrolyzable ATP analogs such as AMPPNP2 are used to accumulate the closed state. Even with AMPPNP, the conformational equilibrium for the cytosolic human (Hsp90α) and the ER Hsp90 (Grp94) still favors the open state (6, 13). Indeed, high temperature is often required to enhance Hsp90α and Grp94’s ATPase to measurable levels (3, 14). This difficulty led to early debate about whether Grp94 is in fact an ATPase (15). Despite this weak activity in vitro, the cellular function of Hsp90 is highly sensitive to ATP competitive inhibitors such as radicicol and geldanamycin.

Many labs are actively testing models of Hsp90’s ATP-driven conformational cycle. Key questions include (i) how Hsp90’s conformational changes are coupled to client protein folding and activation, and (ii) how Hsp90’s conformational cycle is modulated by co-chaperones and posttranslational modifications. These central mechanistic questions rely entirely on the validity of Hsp90’s underlying ATPase cycle measured in isolation.

Detailed biochemical and biophysical experiments that provide the foundation of Hsp90’s conformational cycle have been performed in vitro under dilute conditions. These range from

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2 The abbreviations used are: AMPPNP, 5′-adenylyl-β,γ-imidodiphosphate; ER, endoplasmic reticulum.
FRET and electron microscopy experiments with ~0.01 mg/ml protein to small angle x-ray scattering with up to 10 mg/ml protein. In contrast to in vitro experiments, the cell is a densely crowded system (300–400 mg/ml of macromolecules). In some instances, macromolecular crowding can significantly perturb protein structure and function (16). The impact of crowding on protein behavior should be particularly strong for macromolecules that undergo conformational changes that significantly alter their dimensions. For example, phosphoglycerate kinase activity, which requires an open/closed structural change, is strongly enhanced by crowding (17). Hsp90’s large structural changes motivate an investigation of its sensitivity to molecular crowding.

Protein folding research has revealed hallmarks of crowding on thermodynamic stability (16). Three of these hallmarks are: (i) crowding should become increasingly influential with increasing concentration; (ii) the thermodynamic impact of volume restriction should be entropic; and (iii) crowders of different size should have different potencies, depending on how much volume around the protein is accessible to the crowder in the folded and unfolded states. These expected behaviors all require that the crowder’s effects do not arise from a binding interaction.

The third hallmark listed above highlights a difference between macromolecular crowding for protein folding and crowding as it relates to Hsp90. Although proteins certainly expand upon unfolding, no unfolded structure is known that can be used to determine how much volume is accessible to crowders of different size. Instead, assumptions about the structure of the unfolded state are necessary for such analysis (18). In contrast, for Hsp90, open and closed structures are known, which means that the excluded volume for crowders of different size can be directly calculated. For example, Fig. 1A shows open and closed structures of Hsp90, which visually suggest that the open state may exclude more volume than the closed state.

To test the plausibility of this idea, we performed structure-based calculations (see “Experimental Procedures”). The volume excluded from a crowder increases for both the open and the closed structures as the probe radius increases; however, the open state leaves more volume inaccessible to the probe than the closed state (Fig. 1B). In other words, from the perspective of a large crowder, Hsp90 occupies a larger total volume in the open configuration when compared with the closed configuration. In tightly packed environments, this differential excluded volume provides a strong driving force to minimize the volume occupied by macromolecules. Therefore, crowding should favor the closed hydrolytically active state of Hsp90 over the open state. Because this effect varies with probe size (Fig. 1C), we might anticipate that large crowders should be more potent activators of Hsp90 than small crowders.

Experimental Procedures

The purification of HtpG and Hsp82, Hsp90α, Grp94, BiP, and aha1 has been described previously (6, 13, 19). Briefly, Hsp90 proteins were expressed in Escherichia coli strain BL21* at 37 °C in LB culture medium and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside. Cells were collected through centrifugation and lysed by sonication with 1 mM PMSF. The supernatant was collected and loaded onto a nickel-nitrilotriacetic acid column. Unbound proteins were removed with 20 mM imidazole. Eluted proteins were then purified by anion exchange chromatography on a MonoQ column (GE Health-
Crowding Activates Hsp90

The ATPase activity of bacterial Hsp90 (HtpG) was measured at pH 9, where the chaperone adopts a fully open configuration that undergoes complete closure upon the addition of AMPPNP (8). HtpG activity is greatly enhanced with increasing concentrations of a large polymer crowder (PEG 20,000: radius of gyration, \( R_g \), of 66 Å). The concentration dependence (Fig. 2A) shows a non-saturating increase of activity with an upward curvature. This behavior is a hallmark of crowding via volume exclusion (16). In contrast, the bacterial Hsp70 (DnaK) and Hsp60 (GroEL) chaperones have ATPase activities that are unaffected by PEG 20,000 (Fig. 2A). Similar results with GroEL and DnaK were observed with a different PEG (PEG 3350) and a different crowder (dextran 6000, data not shown). This suggests that Hsp90’s sensitivity to crowding results from the unique large-scale conformational changes that are required for its activity.

To explore the origin of Hsp90’s sensitivity to crowding agents, we tested a series of PEG sizes ranging from the monomer (ethylene glycol) to PEG 33500 (\( R_g \) of 90 Å). These experiments reveal a striking relationship between the physical size of the PEG polymer and the degree to which it activates HtpG (Fig. 2B). Ethylene glycol has a minimal effect, whereas increasing polymer sizes showed increasing activations at the same mass per unit volume. Indeed, at a fixed PEG concentration of 100 mg/ml, the ATPase is increasingly activated for polymers with increasing radius of gyration up to 25 Å. Polymers larger

\[ V_{shell} = V_{A} - V_{1.4} \]

where the volume subscript refers to the probe radius. The fractional excluded solvent volume, \( \Delta V_{crowd} \), is quantified by

\[ \Delta V_{crowd}(r) = (V_{A} - V_{1.4})/V_{shell} \]

where \( r \) is the probe radius.

The activation enthalpy for HtpG was calculated by a linear fit of \( \ln(\text{ATPase}) \) when plotted against the reciprocal of the temperature.

\[ \ln(\text{ATPase}) = A - \Delta H^*/RT \]

where \( \Delta H^* \) is the activation enthalpy, \( R \) is the gas constant, and \( T \) is the temperature in Kelvin.

PEG osmotic pressure data were taken from the literature (21). For concentrations that were not explicitly measured, values were determined via interpolation. PEG radius of gyration was calculated with the following equation (22).

\[ R_g = 0.287 \times M^{0.55} \]

where \( M \) is the molecular weight of the PEG polymer.

Results

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than this size show a uniformly strong activation (Fig. 2C). This experimental relationship, which mirrors the excluded volume relationship calculated for open and closed Hsp90 structures (Fig. 1C), suggests that crowding, by favoring the closed state, increases the chaperone’s ATPase activity as predicted from structures of these two conformations.

Although crowding by excluded volume is a likely driving force behind the increase in HtpG activity, PEG polymers also alter solvent osmotic pressure. An alternative explanation for the PEG size dependence in activating HtpG could be a correlation with the PEG effect on osmotic pressure. To test this possibility, we replotted the activity data from Fig. 2B against the corresponding osmotic pressure by each of the PEGs at their respective concentrations (Fig. 3A); however, no meaningful correlation is observed. Specifically, the smallest PEGs exert the largest effect on osmotic pressure, but have the least influence on activity. Similarly, small-molecule osmolytes and standard cosolvents fail to stimulate HtpG ATPase activity (Fig. 3B). PEGs did not significantly change the ATPase \( K_m \) value (796 and 895 \( \mu M \) with 0 and 100 mg/ml PEG 3350, respectively), which indicates minimal alteration of nucleotide affinity.

The above results suggest that volume exclusion favors the closed, hydrolytically active state of HtpG, and the size dependence of PEG activation can be rationalized from the open and closed structures. The size dependence observed for PEGs (Fig. 2C) should hold for any non-interacting polymer crowder, so we measured HtpG activity with dextran polymers of increasing size. A similar size dependence was indeed observed (Fig. 3C). However, the dextran monomer itself (dextrose) decreased HtpG activity in contrast to the PEG monomer, which showed a minimal activation as expected (Fig. 2B). An appropriate polymer for examining macromolecular crowding should be built from a non-perturbing monomer unit; we therefore used PEG polymers for all subsequent experiments.

All Hsp90s studied to date have a conserved set of conformational states with a species-specific equilibrium between states (13). This suggests that activation by crowding should be conserved, although the magnitude of the response may be variable. Therefore, we measured the PEG dependence of ATPase activation for the yeast Hsp82, human Hsp90\( \alpha \), and mouse Grp94. PEG 20,000 was used for these comparison experiments because this PEG is within the saturation region in Fig. 2C. In all cases, PEG 20,000 at 100 mg/ml produces a large degree of activity stimulation (Fig. 4A). Grp94, which shows the largest increase (15-fold), exhibits a very strong upward curvature with PEG concentration (Fig. 4B).

The strong activation of Grp94 by crowding raises a question as to whether other ER chaperones are equally sensitive to crowding. BiP is an Hsp70-type chaperone specific to the ER. Like Grp94, BiP has a weak ATPase rate limited by ATP hydrolysis (23); however, unlike Grp94, BiP ATP hydrolysis is stimulated by J-proteins, and ADP release is then catalyzed by nucleotide exchange factors (24).

**FIGURE 2. PEG polymers activate HtpG.** A, HtpG ATPase (black circles) increases with increasing concentration of PEG 20,000 (\( R_g \) of 66 Å). The dashed line illustrates the upward-curving relationship between hydrolysis rate and crowder concentration. Both DnaK (blue diamonds) and GroEL (orange triangles) are not activated by PEG. B, crowding activation of HtpG depends strongly on PEG molecular weight. A slice through the PEG concentration series at 100 mg/ml (gray dashed line) shows increasing activation with increasing polymer size. The PEG activation is fully inhibited by 20 \( \mu M \) radicicol (rad). C, ATPase values at 100 mg/ml PEG increase as the \( R_g \) of the crowder approaches 25 Å, but show only modest changes above that size. Buffer conditions were: 25 mm Tris, pH 9.0, 50 mm KCl, 5 mm MgCl\( _2 \), 2 mm ATP, 25 °C. Error bars indicate the S.E. for at least three measurements.
activity of BiP is unchanged by crowding (Fig. 4B). Similar to the findings in Fig. 2A, this result indicates that crowding sensitivity is specific to Hsp90 class chaperones.

**Crowding Activates Hsp90 by Entropic Destabilization of the Open State**—The above results point to a mechanism by which volume exclusion specifically activates Hsp90 by favoring the closed state over the open state. Simple models of crowding predict an entropic thermodynamic driving force. In contrast, if the activation is even partially a result of direct binding of the crowder, then enthalpic contributions should be observed. Therefore, we sought a method to determine the thermodynamic contributions to crowding activation of HtpG.

The temperature dependence of activity can dissect entropic and enthalpic contributions to crowding. We measured HtpG activity over temperatures from 25 to 39 °C with 0, 60, 80, and 100 mg/ml PEG 3350. As is characteristic for an Eyring analysis, the linear correlation between ln(ATPase) and 1/T indicates a single activation enthalpy (Fig. 5). The slope, equivalent to an activation enthalpy of ~10 kcal/mol, is independent of PEG concentration.

**FIGURE 3.** A, PEG activation of HtpG does not correlate with PEG increases in osmotic pressure, cP, centipoise. B, cosolvents and osmolytes do not show crowding-like activation of HtpG. C, ATPase values with dextran polymers at 100 mg/ml follow a similar size dependence as PEG polymers. The solid line is a visual guide. Buffer conditions were: 25 mM Tris, pH 9.0, 50 mM KCl, 5 mM MgCl₂, 2 mM ATP, 25 °C.

**FIGURE 4.** A, 100 mg/ml PEG 20,000 increases the activity of multiple Hsp90 homologs. The most dramatic ATPase enhancement is observed for Grp94, increasing the rate by a factor of 15. ATPase activity for all homologs is completely inhibited by radicicol (rad). B, Grp94 displays a strong upward-curving relationship between hydrolysis rate and crowder concentration (red circles). No ATPase activity is observed in the presence of radicicol (blue triangles). The Hsp70 class chaperone BiP is not activated by PEG (black diamonds). Buffer conditions were as described under “Experimental Procedures.” Error bars indicate the S.E. for at least three measurements.

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Crowding Activates Hsp90

The natural logarithm of HtpG ATPase in PEG 3350 (0 mg/ml, red circles; 60 mg/ml, blue squares; 80 mg/ml, green diamonds; 100 mg/ml, black triangles) is plotted as a function of 1/T. The linear fit indicates a single constant activation enthalpy (ΔH°, see “Experimental Procedures”). The fit value of ΔH° is independent of PEG concentration (inset). Buffer conditions were: 25 mM Tris, pH 9.0, 50 mM KCl, 5 mM MgCl₂, 2 mM ATP. Error bars indicate the S.E. for at least three measurements.

FIGURE 5. Eyring analysis reveals entropic activation of HtpG by PEG crowders. The natural logarithm of HtpG ATPase in PEG 3350 (0 mg/ml, red circles; 60 mg/ml, blue squares; 80 mg/ml, green diamonds; 100 mg/ml, black triangles) is plotted as a function of 1/T. The linear fit indicates a single constant activation enthalpy (ΔH°, see “Experimental Procedures”). The fit value of ΔH° is independent of PEG concentration (inset). Buffer conditions were: 25 mM Tris, pH 9.0, 50 mM KCl, 5 mM MgCl₂, 2 mM ATP. Error bars indicate the S.E. for at least three measurements.

Concentration. We thereby infer that the crowding activation of HtpG is entropically driven.

How does crowding change the Hsp90 energy landscape? The above measurements do not yet demonstrate whether crowding exerts its energetic influence on the open or closed side of the landscape. Previous work established that closure is rate-limiting in Hsp90’s ATPase activity (11, 12). Therefore, we expect that crowding should increase the single-step rate of closure. In contrast, if crowding activation were driven by a change in the oligomeric state of Hsp90, or if crowding allowed the open state to hydrolyze ATP, then no change in closure rate would be expected.

We tested these possibilities by measuring the rate of HtpG closure using a previously established FRET assay in which the donor and acceptor fluorophores are labeled on opposite arms. Closure is initiated by adding AMPPNP, and an increase in transfer efficiency is observed over time (11, 12). The addition of PEG accelerates closure kinetics (Fig. 6A). The closure rate measured by FRET (0.001 s⁻¹ in the absence of PEG) shows a non-saturating increase, similar to that observed for ATPase measurements (Fig. 6C). Interestingly, the relative increase in closure kinetics is greater than the relative increase in the ATPase. This raises the intriguing possibility that at sufficiently high concentrations of crowding, Hsp90 could have a different rate-limiting step in its ATPase cycle.

Closure acceleration by crowding indicates that the closure transition state is compact relative to the open state. The degree of compaction in the transition state is fundamentally important for understanding energy consumption by Hsp90. Therefore, a critical distinction is whether crowding affects the rate of reopening after closure. If crowding slows the reopening rate, then the transition state has an intermediate degree of compaction relative to the closed state. If reopening is not affected by crowding, then the transition state has similar compaction as the closed state. Reopening kinetics were measured by first pre-closing HtpG and then adding an excess of ADP and measuring the loss of FRET efficiency (12, 25). Reopening rates (0.002 s⁻¹ in the absence of PEG) are slightly faster than closure (12), suggesting that full reopening may not be required for nucleotide exchange. PEGs have no influence on the measured reopening rate (Fig. 6, B and C). These findings suggest that the transition state lies far toward the closed conformation, and crowding acts via destabilizing the open configuration (Fig. 6D).

Crowding Activation of Hsp90 Is Independent of Co-chaperone Activation—The energy landscape picture that emerges is that crowding entropically destabilizes the open configuration of Hsp90, and therefore decreases the energy barrier to the active closed state. A crucial distinction is whether crowding works by the same mechanism or a different mechanism of other known activators. The non-saturable increase in activity by PEGs is different from the saturable increases in Hsp90 activity that have been observed for co-chaperones and substrate proteins. This suggests that crowding could operate in an additive manner with the activation resulting from co-chaperone or substrate protein interactions.

Aha1 is a well established activating co-chaperone of Hsp90 (26, 27). Fig. 7A illustrates a saturating effect of aha1 on the Hsp82 ATPase. If crowding works by the same mechanism, then no additional activation by PEGs would be expected. In contrast, if crowding works by a different mechanism, then an additional increase of activity would be expected. Indeed, we observe that PEG 3350 and aha1 together yield a greater activation than aha1 alone (Fig. 7A). We interpret this result with an energy landscape in which crowding and co-chaperone activating mechanisms operate independently (Fig. 7B). The effective affinity (apparent Kₘ) of aha1 for Hsp82 is increased by adding PEG, which indicates that PEG does not compete for aha1 binding sites on Hsp82. We interpret these results in terms of an energy landscape in which the transition state stabilization from aha1 is independent of the open state destabilization by crowders (Fig. 7B).

Discussion

In this study, we establish that molecular crowding activates Hsp90, whereas the activities of Hsp60 and Hsp70 class chaperones are insensitive to these conditions. We have used polymers as tractable models for the crowded cellular environment. Our findings highlight a strong sensitivity of Hsp90’s structure and activity to crowding. Our results suggest that important mechanistic differences in Hsp90 behavior could be masked in studies done under conventional dilute in vitro conditions.

Polymer crowders elicit increasing activation with increasing concentration (Fig. 2A). This non-saturating concentration dependence is characteristic of crowding in other contexts (16). The degree of Hsp90 activation is strongly dependent on the size of the polymer crowder (Fig. 2, B and C) and can be recapitulated from simple calculations comparing the excluded volume of the open and closed configurations of Hsp90 (Fig. 1C).
Despite this agreement, the volume of static structures can only provide a limited understanding of why crowding activates Hsp90.

A simple explanation of these results is that crowding activates Hsp90 because Hsp90’s open state occupies more volume than the closed state from the perspective of a large crowder. If true, the driving force behind the crowding activation of Hsp90 should be entropic destabilization of the open state relative to the closed state. This proposed entropic driving force is supported by the measured activation enthalpy remaining constant with increasing PEG (Fig. 5). Our findings are not consistent with a model in which Hsp90 is activated by binding crowding agents.

The energy landscape picture that emerges from these results is that crowding destabilizes the open state relative to the closed state and the transition state. This picture demands that crowding should accelerate Hsp90 closure but not reopening. Both of these expectations are observed with kinetic FRET measurements (Fig. 6). Our results imply that the closure transition state structure has a similar degree of compaction as the closed state itself.

In contrast to crowding destabilization of the open state, activators of Hsp90, such as aha1, can stabilize the transition state relative to the open state (26). This difference in the underlying mechanism is reflected in the additive effects of crowding to the ATPase activation of Hsp82 by aha1 (Fig. 7). A key open question is how crowding affects interactions between Hsp90 and its client proteins.

Crowding activates many different Hsp90 homologs, with the ER-specific Hsp90, Grp94, showing the highest fold activation (Fig. 4). Diffusion measurements have shown that the ER can be significantly more crowded than the cytosol (28). The ER experiences large fluctuations in secretory flux, and control mechanisms allow the ER to regulate its size to match secretion burden (29). It is interesting to speculate that Grp94’s activity could be tuned by the extent of crowding within the ER. Future work is needed to identify the molecular origin of Grp94’s enhanced response to crowding. Crowding activation again appears to be unique to Hsp90’s large-scale conformational changes that are required for its activity, as similar measurements on the BiP chaperone do not show increased activity.

In many cases, the effects of cellular crowding on protein folding are modest (30, 31), and it is important to draw a distinction between crowding as it relates to protein folding versus the strong effects observed for Hsp90. In folding, volume exclusion favors the more compact native state relative to the

**FIGURE 6. PEG crowders accelerate HtpG arm closure.**

A, FRET kinetic analysis reveals that HtpG arm closure rate increases with increasing PEG 3350 (0 mg/ml, black circles; 60 mg/ml, blue; 100 mg/ml, green; 150 mg/ml, red). Solid lines are fits to single exponential kinetics. The inset shows the open and closed states with the fluorescently labeled sites highlighted in red and blue. B, HtpG arm reopening rate is unchanged with increasing PEG 3350 (0 mg/ml, black circles; 60 mg/ml, blue; 100 mg/ml, green; 150 mg/ml, red). Solid lines are fits to single exponential kinetics. C, the PEG concentration series displays an upward-curving relationship between arm closure rate (circles) and crowder concentration. In contrast, reopening rates (squares) are PEG-independent. Error bars indicate the S.E. for at least three measurements. D, FRET observations are consistent with an Hsp90 energy landscape wherein crowding destabilizes the open conformation, thereby increasing closure rate and leaving reopening rate unchanged.
Crowding Activates Hsp90

![Graph A](image)

**FIGURE 7.** A, Hsp82 is activated by increasing concentrations of the aha1 co-chaperone (red circles). The addition of 60 mg/ml PEG 3350 increases the maximal activity and effective binding constant (blue squares). Solid lines are fits to the Michaelis-Menten equation. B, an energy landscape interpretation in which crowding destabilizes the open configuration and aha1 stabilization of the transition state can act in an additive manner. Buffer conditions were: 25 mM Tris, pH 9.0, 12 mM KCl, 5 mM MgCl₂, 2 mM ATP, 25 °C. Error bars indicate the S.E. for at least three measurements.

Expanded unfolded state, in cases where the crowder is non-interacting (18). However, in situations where crowding involves both volume exclusion and weak binding interactions, then unfolded configurations are often stabilized from their greater propensity to make weak nonspecific interactions. The consequences of these opposing forces are only modest changes in net stability for proteins in vitro versus crowding conditions in the heterogeneous cellular environment (31, 32).

In contrast to protein folding, Hsp90 presents a folded surface to crowders in both the open and the closed configurations, which suggests that weak Hsp90/crowder interactions will not favor either state. Indeed, both polymer and protein crowders activate Hsp90, although the extent of activation is variable for protein crowders (data not shown). We only observed decreased activity with viscous solvents (glycerol and dextrose) and a denaturant (urea).

Previous work demonstrated that osmolytes affect the conformational state of Hsp90, but have little influence on its ATPase activity (33). Work from Auton and Bolen (34) has suggested that the energetic influence of osmolytes originates primarily from the exposure of the peptide backbone. Our findings here separate these surface area-driven effects from volume exclusion-driven effects.

Together our results suggest that Hsp90 may have a much higher intrinsic activity in the cell than in vitro. Tremendous effort has been invested in characterizing Hsp90 structure and conformational dynamics in vitro. More work is now needed to test the degree to which the in vitro Hsp90 conformational cycle matches its behavior in vivo.

**Author Contributions**—J. C. H. performed FRET experiments. B. H. performed activity measurements in Figures 2, 3, 4, and 5. M. S. performed aha1 activity measurements in Figure 7. T. O. S. and J. C. H. conceived the idea of the project and wrote most of the manuscript.

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