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Conformational dynamics of the molecular chaperone Hsp90

Kristin A. Krukenberg[1,3], Timothy O. Street[1], Laura A. Lavery[1] and David A. Agard[1,2]*

[1] Department of Biochemistry and Biophysics, University of California, San Francisco, CA, USA
[2] The Howard Hughes Medical Institute, University of California, San Francisco, CA, USA
[3] Present address: Department of Systems Biology, Harvard Medical School, Boston, MA, USA

Abstract. The ubiquitous molecular chaperone Hsp90 makes up 1–2% of cytosolic proteins and is required for viability in eukaryotes. Hsp90 affects the folding and activation of a wide variety of substrate proteins including many involved in signaling and regulatory processes. Some of these substrates are implicated in cancer and other diseases, making Hsp90 an attractive drug target. Structural analyses have shown that Hsp90 is a highly dynamic and flexible molecule that can adopt a wide variety of structurally distinct states. One driving force for these rearrangements is the intrinsic ATPase activity of Hsp90, as seen with other chaperones. However, unlike other chaperones, studies have shown that the ATPase cycle of Hsp90 is not conformationally deterministic. That is, rather than dictating the conformational state, ATP binding and hydrolysis only shift the equilibria between a pre-existing set of conformational states. For bacterial, yeast and human Hsp90, there is a conserved three-state (apo–ATP–ADP) conformational cycle; however; the equilibria between states are species specific. In eukaryotes, cytosolic co-chaperones regulate the *in vivo* dynamic behavior of Hsp90 by shifting conformational equilibria and affecting the kinetics of structural changes and ATP hydrolysis. In this review, we discuss the structural and biochemical studies leading to our current understanding of the conformational dynamics of Hsp90, as well as the roles that nucleotide, co-chaperones, post-translational modification and substrates play. This view of Hsp90's conformational dynamics was enabled by the use of multiple complementary structural methods including, crystallography, small-angle X-ray scattering (SAXS), electron microscopy, Förster resonance energy transfer (FRET) and NMR. Finally, we discuss the effects of Hsp90 inhibitors on conformation and the potential for developing small molecules that inhibit Hsp90 by disrupting the conformational dynamics.

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* Author for correspondence: D. A. Agard, Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94158, USA.
Tel.: 415-476-2521; Fax: 41-476-1902; Email: agard@msg.ucsf.edu

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1. Introduction

Hsp90 is a highly conserved member of the class of proteins known as molecular chaperones, which promote protein folding by reducing misfolding and aggregation. Well-known chaperones such as Hsp60 (GroEL) and Hsp70 (DnaK) interact with unfolded polypeptide chains and reduce misfolding through interactions with hydrophobic surfaces (Gomez-Puertas *et al.* 2004; Kurt *et al.* 2006; Rudiger *et al.* 1997; Weissman *et al.* 1995). These chaperones undergo rounds of ATP hydrolysis causing conformational changes that are coupled to substrate release into the cytosol by Hsp70 or into the lumen of Hsp60 where proper folding can occur (Walter & Buchner, 2002).

Similarly, Hsp90 can bind and hydrolyse ATP and is believed to interact with hydrophobic surfaces on substrate (client) proteins. Indeed, like other molecular chaperones Hsp90 can suppress protein thermal aggregation *in vitro* (Jakob *et al.* 1995; Wiech *et al.* 1992). However, in eukaryotes Hsp90 plays a unique role among chaperones by its important regulatory effects. These include the activation of kinases and steroid hormone receptors, the formation of protein–protein complexes and effects on trafficking and turnover, all of which involve diverse complexes with clients, co-chaperones and binding partners of clients (Pearl & Prodromou, 2006). In addition to these interactions with specific client classes, Hsp90 also is involved in general *de-novo* folding by receiving nascent unfolded proteins transferred from Hsp40/70 through bridging interactions with Hop (Hsp70/Hsp90 organizing protein). Hsp70 is known to bind short linear hydrophobic peptides, and so the transfer to Hsp90 suggests that clients may bind after early folding steps that sequester these exposed hydrophobic regions by the formation of secondary and super-secondary structures. Indeed, Hsp90 is generally understood to act in the late stages of folding (Jakob *et al.* 1995).

The role of Hsp90 in late-stage folding is consistent with co-chaperone-recruited clients such as kinases and steroid hormone receptors that likely have already achieved a largely folded or almost fully folded conformation upon interacting with Hsp90 (Jakob *et al.* 1995; McLaughlin *et al.* 2002). Subsequent ATP-dependent conformational changes within Hsp90 are believed to induce client conformational rearrangements that facilitate the binding of ligands or partner

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Table 1. *Kinetic parameters of ATP hydrolysis for homologs of Hsp90 at 25 °C*

Homolog	K_m (μM)	ATPase rate (s^{-1})
Human Hsp90[a]	190	0·0009
Yeast Hsp90[b,f]	100	0·02
HtpG[c,f]	250	0·011
Trap1[d]	15·4	0·0027
Grp94[e]	92	0·006

Parameters were taken from the following: (a) McLaughlin *et al.* (2004), (b) Weikl *et al.* (2000), (c) Graf *et al.* (2009), (d) Leskovar *et al.* (2008), (e) Frey *et al.* (2007), (g) Cunningham *et al.* (2008) and (f) measured at 30 °C.

proteins (Richter & Buchner, 2001; Young *et al.* 2001; Zhao *et al.* 2005). A list of *in vivo* identified Hsp90 clients (http://www.picard.ch) indicate no shared sequence or structural characteristics, and span a remarkable size range (e.g. α-synuclein to telomerase, 14–290 kDa (Falsone *et al.* 2009; Forsythe *et al.* 2001)). This client diversity immediately raises the question of how Hsp90 can achieve such broad specificity. As discussed in this review, Hsp90's conformational flexibility likely plays a central role.

Particular Hsp90 clients, such as p53, Cdk4, c-Src/v-Src and HER-2/HER-3, are oncogenic or otherwise required for cell proliferation, making Hsp90 an attractive drug target (Neckers, 2007). Inhibition of Hsp90 with small molecules such as geldanamycin (GA) and its derivatives has been shown to reduce tumour growth, and several of these compounds are currently in clinical trials (Chiosis *et al.* 2003; Neckers & Ivy, 2003; Solit *et al.* 2008; Workman, 2004). Hsp90 has also been implicated in other diseases including Alzheimer's (Dickey *et al.* 2008), vascular disease (Shah *et al.* 1999) and viral diseases (Geller *et al.* 2007). Although the role of Hsp90 in such diverse disease processes makes it a promising drug target, the full utility of Hsp90 inhibitors is clearly limited by the lack of molecular-level information about client binding and the role of ATP hydrolysis.

Hsp90 has been shown to be a highly flexible and dynamic molecule, and large conformational changes are coupled to ATP binding and hydrolysis. All Hsp90 homologs studied so far exhibit slow ATPase activity and this activity is essential for the correct functioning of Hsp90 in the cell. The ATPase activity varies substantially between homologs (Table 1), but precise regulation of the ATPase rate appears essential. Mutant Hsp90s with either a faster or slower ATPase rate result in temperature-sensitive growth phenotypes and are unable to fully activate *in vivo* clients such as the glucocorticoid receptor (GR) (Nathan & Lindquist, 1995; Prodromou *et al.* 2000). Although it is clear that the ATPase function of Hsp90 plays an important role in recognizing and activating client proteins, the conformational changes and determinants of client recognition associated with Hsp90 function remain unclear. It is of tremendous importance to understand the various means of regulating the dynamics and therefore function of Hsp90. The exact nature of the conformations of Hsp90, their function and regulation have become an area of intense study in recent years, and, as discussed below, much progress has been made in understanding the conformational dynamics of Hsp90 and the role that these dynamics play in chaperone function.

2. Architecture of Hsp90

Hsp90 is highly conserved from bacteria to eukaryotes with ~50% sequence similarity between *Escherichia coli* and humans. There are four human Hsp90 isoforms: two cytosolic (Hsp90α,

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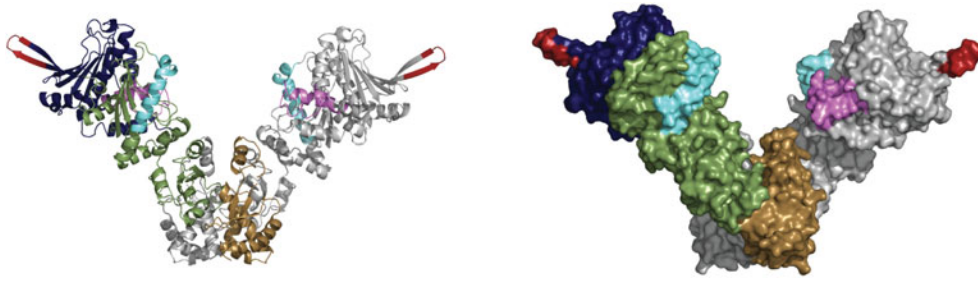


Fig. 1. Hsp90 exists as a homodimer with three domains per monomer. The crystal structure of the bacterial homolog, HtpG (PDB code 2IOQ), is shown as both a cartoon representation and a surface representation. The domains of one monomer are colored blue (NTD), green (MD) and brown (CTD). The other monomer is colored gray. The NTD binds nucleotide and contains a variable charged loop (red) and the conserved lid region (purple), which is adjacent to the nucleotide-binding pocket. The MD contains the conserved catalytic loop (cyan) that is essential for ATP hydrolysis. The CTD provides the constitutive dimer interface.

Hsp90β); one mitochondrial (Trap1); and one specific to the endoplasmic reticulum (Grp94). These isoforms all operate as obligate dimers consisting of three domains per monomer (Fig. 1). The C-terminal domain (CTD, brown) is the site of dimerization, the middle domain (MD, green) has been implicated in client binding, and ATP binds to the N-terminal domain (NTD, blue) (Pearl & Prodromou, 2006). The nucleotide-binding pocket in the NTD is highly conserved across species, and has been identified as related to the gyrase, Hsp90, histidine kinase, MutL (GHKL) superfamily of ATPases (Bergerat *et al.* 1997; Dunbrack *et al.* 1997). As with other GHKL superfamily proteins, binding and hydrolysis of nucleotide leads to local and global conformational changes that result in closure by dimerization of the NTDs.

Within the domains of Hsp90 there are conserved elements that are functionally important in the nucleotide cycle. These elements include the nucleotide lid (Fig. 1, purple region on NTD) and the catalytic loop (cyan region on MD), which will be discussed in detail in the 'ATP-dependent conformational changes' section. The three individual domains of Hsp90 show high levels of structural conservation but the domain boundaries are only weakly coupled, which allows for large structural changes to occur by domain–domain rearrangements.

Despite the conserved domain architecture and key structural elements, there are important differences in the overall domain organization of the different Hsp90s (Fig. 2). A repetitively charged region (Fig. 1, red) that varies in length is present at the C-terminal end of the NTD in yeast and cytosolic higher eukaryotic Hsp90s as well as in Grp94. This charged region is also present in the bacterial protein HtpG, but it is significantly shorter. Although the charged region is dispensable in yeast, it has been implicated in client binding (Scheibel *et al.* 1999). In addition to the charged linker, the yeast and cytosolic higher eukaryotic Hsp90s also have a C-terminal motif (MEEVD) that facilitates co-chaperone binding. The unique regulatory role of Hsp90 in eukaryotes is likely due to the large number of co-chaperones that recruit specific classes of clients (e.g. kinases via p50/Cdc37), modulate the Hsp90 ATPase activity (e.g. activation by Aha1), aid in client folding (e.g. proline isomerization by cyclophilins) and maturation (p23). Many of these co-chaperones contain tetratricopeptide repeat (TPR) domains that bind Hsp90 via the C-terminal MEEVD motif. Later we will discuss the role of co-chaperones on Hsp90 conformational dynamics.

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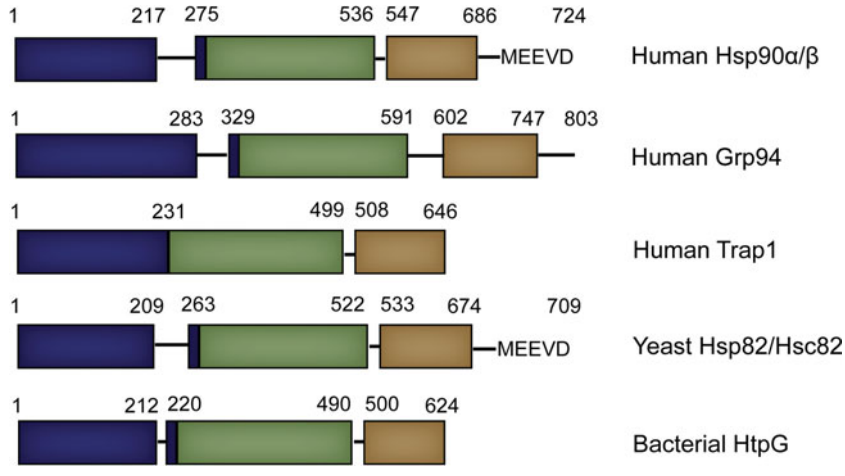


Fig. 2. Domain architecture for different Hsp90 homologs. The domains are colored with the NTD in blue, the MD in green and the CTD in brown.

3. Apo state flexibility of Hsp90

The central importance of Hsp90 to both basic cellular processes and disease states makes Hsp90 an important target for structural studies. After the first crystal structures of the NTD were determined in 1997 by the Pearl and Pavletich labs (Prodromou *et al.* 1997b; Stebbins *et al.* 1997), it would take nearly another decade before full-length Hsp90 structures would be determined: in 2006 by the Pearl lab (AMPPNP conditions) (Ali *et al.* 2006) and in 2007 by the Agard lab (apo and ADP conditions) (Shiau *et al.* 2006). The significant difficulty in determining full-length Hsp90 crystal structures is likely due to its remarkable structural flexibility. For example, under apo conditions the Hsp90 from *E. coli*, HtpG, crystallized in a 'V'-shaped conformation (Fig. 3*a*) (Shiau *et al.* 2006) while in solution SAXS and electron microscopy measurements demonstrated that a highly open conformation and a more closed conformation are populated in a pH-dependent manner (Fig. 3*b*) (Krukenberg *et al.* 2009b). The more closed state is similar to a crystal structure of the Hsp90 homolog specific to the endoplasmic reticulum, Grp94 (Dollins *et al.* 2007). The apo conformations largely differ by rigid-body rotation at the interface between the middle and CTDs, creating a variable-sized cleft between the monomer arms. These conformational changes are dramatic, with the NTDs undergoing 50 Å translations and 50° rotations upon the open/Grp94 transition.

Both SAXS and electron microscopy (EM) measurements have shown that Hsp90 conformations coexist in a delicate equilibrium. That is, for HtpG the open and Grp94 conformation have approximately the same free energy. The weak energetics associated with the conformational equilibrium has been illustrated by the use of a class of small molecules called osmolytes, small molecules that maintain protein homeostasis under harsh environments and stress conditions by their influence on protein folding and stability. SAXS measurements of Hsp90 in the presence of osmolytes demonstrated that the chaperone conformational equilibrium can be effectively dialed between different conformational states over a similar energetic range under which protein folding occurs (Street *et al.* 2010). This suggests that the Hsp90 conformation could be energetically linked to folding steps.

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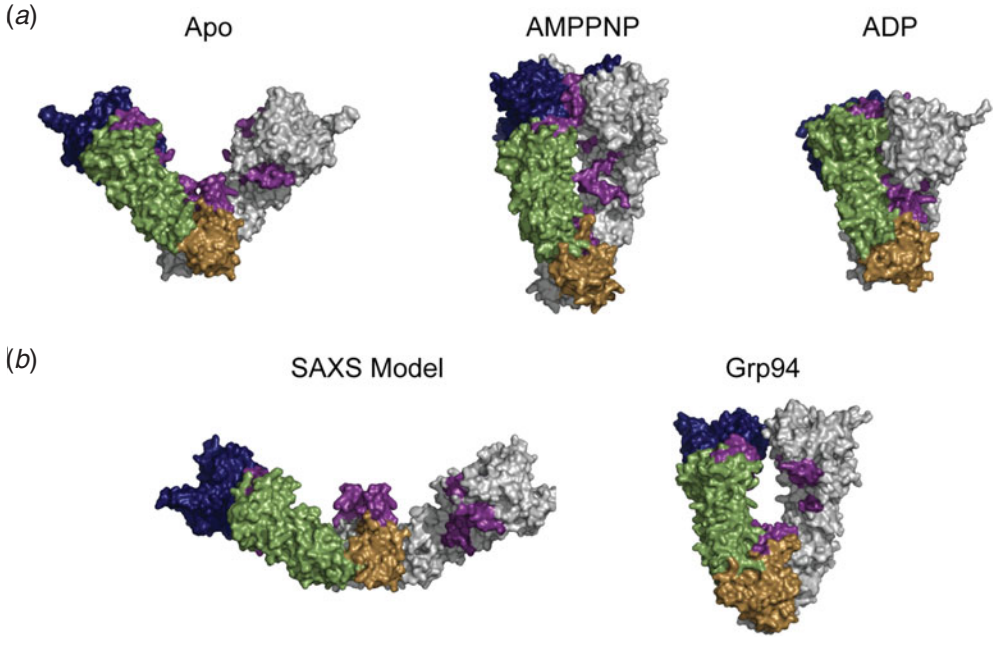


Fig. 3. Full-length structures of Hsp90. The NTD is shown in blue, the MD in green and the CTD in brown. In all structures, exposed hydrophobic surfaces are highlighted in purple. (*a*) Surface representations of the apo and ADP HtpG structure (Shiau *et al.* 2006) and the yeast Hsp90 AMPPNP-bound structure (Ali *et al.* 2006). (*b*) Surface representations of the apo HtpG solution state (Krukenberg *et al.* 2008) and the Grp94 crystal structure (Dollins *et al.* 2007).

The extreme apo state conformational heterogeneity is universal to all homologs that have been examined (HtpG, Hsc82, Hsp90α, Grp94 and TRAP, Krukenberg *et al.* 2009a and unpublished observations). This conservation implies that the flexibility of Hsp90 is functionally important, and that differences in conformational flexibility among Hsp90 homologs may relate to differences in how they interact with clients. If Hsp90 conformational changes are functionally linked to client interactions, two predictions can be made: (1) clients will play an active role in determining the chaperone conformation; and (2) different conformational states of Hsp90 will have different effects on client folding/misfolding.

The first prediction has been observed for three clients: the ligand-binding domain of GR; the Cdk4 kinase; and a model unfolded client Δ131Δ, all of which will be discussed in 'Client protein binding affects Hsp90 conformation'. In summary, the ligand-binding domain of GR stimulates the human Hsp90 ATPase, which indicates that the Hsp90 conformation is being driven towards the closed catalytically active state. For the co-chaperone-recruited Cdk4, an electron microscopy reconstruction shows that binding occurs asymmetrically on Hsp90 and results in incomplete closure. Finally, the model unfolded protein Δ131Δ drives Hsp90 to partially close under apo conditions, and accelerates full closure and ATP hydrolysis under nucleotide conditions. As discussed later, Hsp90's differing response to these very different proteins highlights the remarkable capacity of this chaperone to accommodate structurally diverse clients.

The second prediction, that different Hsp90 conformations will have different effects on client folding/misfolding has also been observed for a model client. Specifically, the thermal

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aggregation of citrate synthase can be strongly suppressed by HtpG in the Grp94 conformation but only weakly suppressed in the open state (Krukenberg *et al.* 2009b). This observation was enabled by identifying a mutation pair (H446E/K) that selectively populates the open/Grp94 conformations independent of pH.

These observations together support the idea that the conformational equilibrium of Hsp90 plays a central role in client interactions, and is possibly tuned to the client requirements of each organism. The large structure of Hsp90 in combination with its conformational diversity suggests that the chaperone can adopt a combinatorial set of binding surfaces and chaperone geometries in order to interact with structurally diverse clients and large complexes that include co-chaperones. As discussed next, the effect of nucleotide binding and hydrolysis adds a layer of complexity, both structural and kinetic, to the Hsp90 conformational equilibrium.

4. ATP-dependent conformational changes

The transition to the closed ATP state involves three local structural changes that together coordinate large-scale closure of the chaperone. ATP binding (1) restructures an N-terminal helical lid region, (2) dramatically changes the NTD/MD orientation and (3) is associated with the release of a β-strand, which is swapped across monomers stabilizing N-terminal dimerization. These changes are discussed below.

The lid region (Fig. 1, purple), a helix–loop–helix motif adjacent to the nucleotide-binding pocket makes extensive NTD dimer contacts (Prodromou *et al.* 1997a; Stebbins *et al.* 1997). Crystal structures of isolated NTDs in the apo, AMPPNP and ADP bound states show that nucleotide binding induces large structural changes in the lid (Fig. 4). Biochemical studies highlight that the lid has a complex role in regulating NTD dimerization and ATP hydrolysis. Specifically, removal of the lid abolishes all ATPase activity, but heterodimers lacking the lid on only one NTD have substantially accelerated closure and ATPase hydrolysis rates (Hessling *et al.* 2009; Richter *et al.* 2006). The increased dimerization promoted by this heterodimer is coupled to the first 24 amino acids of the N-terminus that form a swapped β-strand that stabilizes NTD dimerization. Deletion of the N-terminal 24 residues confers flexibility to the lid suggesting that nucleotide binding, lid restructuring and β-strand swapping are all coupled (Richter *et al.* 2006).

A second major structural change upon ATP binding is a repositioning of the NTD/MD interface, which orients a highly conserved arginine (R380, in yeast numbering) that resides on the MD catalytic loop (Fig. 1, cyan) to interact with the ATP γ-phosphate. In the AMPPNP-bound structure of Hsp90, R380 is the only residue contacting the γ-phosphate, highlighting its unique role in ATP binding and hydrolysis. The catalytic loop and arginine are both highly conserved and appear essential for hydrolysis by Hsp90 alone and for the acceleration of hydrolysis by the Aha1 co-chaperone (Meyer *et al.* 2003). The NTD/MD orientation has been found to be variable in different crystal structures, suggesting that rotational freedom allows for the R380/γ-phosphate alignment to be regulated by co-chaperone and client binding. The R380 residue on the catalytic loop forms a hydrophobic network with a loop (T22, V23 and Y24, Hsp82 numbering) on the opposing monomer in the closed state, indicating cross-monomer coupling of closure and hydrolysis (Fig. 5). Indeed, cross-monomer regulation of hydrolysis was confirmed by examining the ATPase activity of a series of yeast Hsp90 heterodimers with mutations in this network (Cunningham *et al.* 2008). The catalytic role of the MD and the requirement for cross-monomer interactions in hydrolysis is further supported by truncation experiments where Hsp90 deletion constructs lacking the CTD retain basal ATPase activity if the

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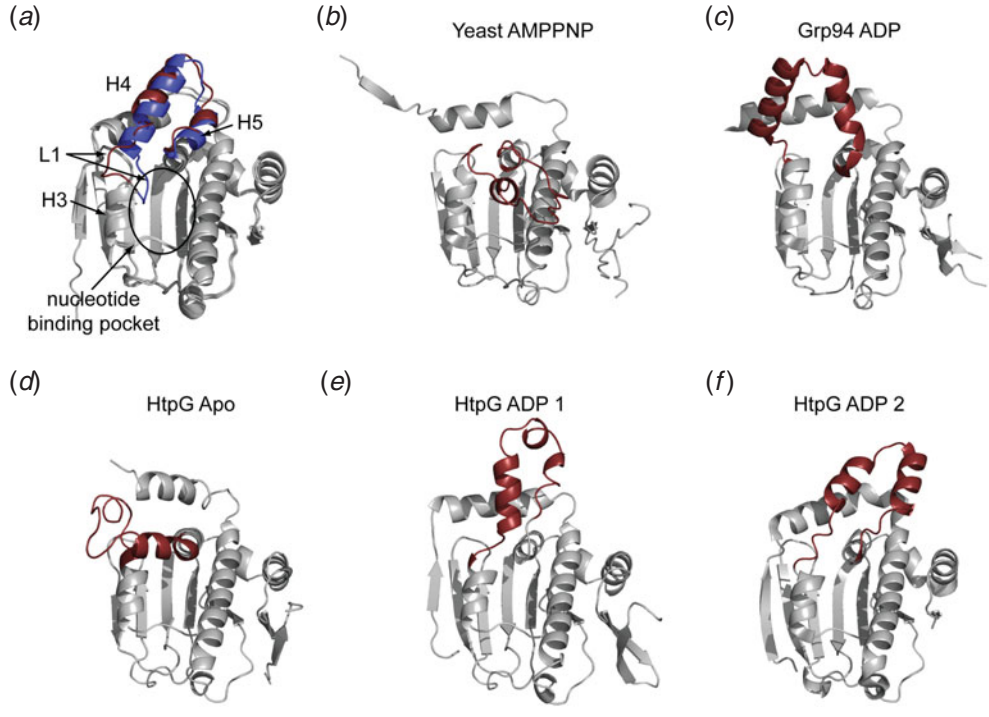


Fig. 4. Variation in the NTD of Hsp90 occurs mostly in the lid. All structures are of the NTD of Hsp90 with the lid highlighted in red or blue. (*a*) Two conformations of the isolated human Hsp90 NTD (1YER and 1YES). In one conformation (blue) loop 2 (L2) extends into the nucleotide-binding pocket. Large motions in the lid can also be seen in (*b*) the NTD from the full-length yeast structure (2CG9) and (*c*) the isolated NTD of Grp94 bound to ADP (1TBW). (*d–f*) Three additional lid conformations are observed in crystals of HtpG (2IOR, 2IOQ and 2IOP).

two monomers are connected by a linker, but cross-linked NTDs lacking the MD exhibit 100-fold lower ATPase activity than WT (Wegele *et al.* 2003).

ATP binding by Hsp90 results in local conformational changes that drive closure leading to a state where the hydrophobic surfaces are less well exposed than in the apo state but still accessible on the outer edges of the dimer cleft (Fig. 3*a*, purple). The closed conformation of Hsp90 was determined crystallographically for the yeast homolog in complex with the co-chaperone p23, which binds the NTD. Subsequent SAXS and EM reconstructions demonstrated that the closed state is populated by the bacterial and human Hsp90 homologs as well, although the extent to which these homologs populate the closed state is quite variable (yeast > bacterial > human). This ranking matches the Hsp90 homolog ranking of ATP hydrolysis rates (Table 1), suggesting that closure is the rate-limiting step in hydrolysis. Indeed, yeast Hsp90 variants with decreased hydrolysis rates show decreased closure (Cunningham *et al.* 2008). However, the rate-limiting structural steps in closure are currently unknown.

The connection between the slow hydrolysis rate of Hsp90 and closure to the ATP state is also supported by FRET measurements showing slow closure kinetics similar to measured ATPase rates (Hessling *et al.* 2009). The FRET-measured closure kinetics are multiphasic, indicating intermediates. In support of this, hydrogen–deuterium exchange mass spectrometry (HX-MS) experiments have demonstrated that an intermediate conformation, with increased catalytic loop protection, exists before the closed state in the hydrolysis reaction (Graf *et al.* 2009). Mutation

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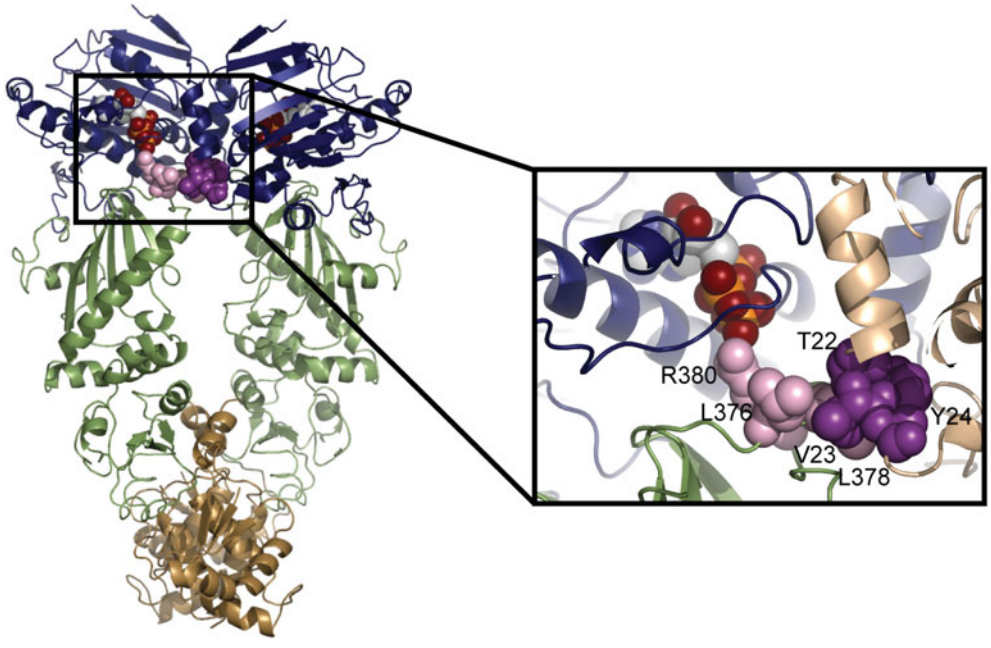


Fig. 5. A hydrophobic interaction network is formed between the MD catalytic loop and the NTD of opposing monomers. The NTD is shown in blue, the MD in green and the CTD in brown. ATP and the residues involved in the interaction network are shown as spheres with residues from the NTD shown in dark purple and residues from the MD shown in lavender. In the expanded view, the second monomer is shown in beige.

experiments also indicate that the structure of the ATP hydrolysis transition state must be distinct from the yeast AMPPMP:p23 crystal structure. Mutation of T22 to Y significantly accelerates ATP hydrolysis, but would be sterically precluded by the crystal structure (Cunningham *et al.* 2008). Closure kinetics have been measured upon addition of non-hydrolysable or slowly hydrolysable ATP by both SAXS (T. O. Street & D. A. Agard, unpublished results) and FRET (Hessling *et al.* 2009), whereas in the presence of ATP an open, low FRET, conformation is maintained. This observation indicates that hydrolysis and subsequent reopening occur quickly compared to the slow, ∼10 min scale, closure reaction. The large range of time scales associated with different steps in the Hsp90 reaction cycle demonstrates how kinetic regulation by co-chaperones can play a pivotal role in affecting client interactions.

In the presence of ADP, a compact structure was observed for HtpG (Fig. 3) (Shiau *et al.* 2006). In this state the exposed hydrophobic surfaces contributed by each domain in the apo and ATP states are buried in the core of the protein suggesting a means for client protein release. Negative-stain EM and cross-linking studies demonstrated the presence of the compact ADP state in HtpG, yeast Hsp90 and human Hsp90 indicating that this state is conserved (Southworth & Agard, 2008). The ADP-state conformation is also consistent with cross-linking studies of Grp94 (Chu *et al.* 2006), in which the observed cross-links are only compatible with the compact conformation. However, for human and yeast Hsp90 the ADP state is only observed in the presence of cross-linker, highlighting the transient nature of the conformation. This is also evident from SAXS measurements that show a minimal change in the bulk population under ADP conditions (Krukenberg *et al.* 2008). Together, the structural information on Hsp90's

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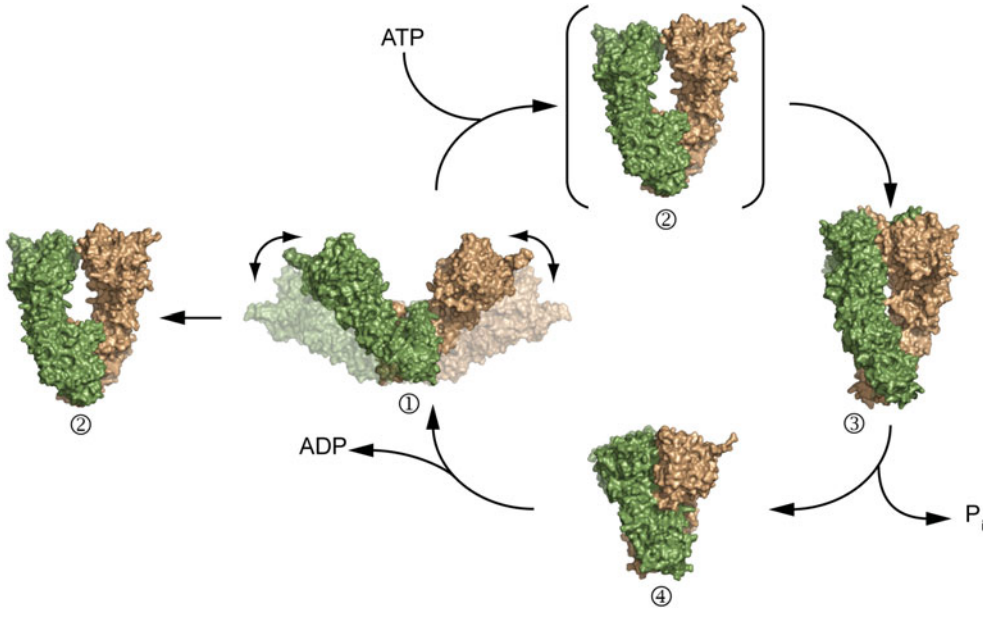


Fig. 6. The structures of Hsp90 suggest a nucleotide-dependent conformational cycle. In this cycle, the binding of ATP shifts the conformation from an open structure (1) to an NTD-dimerized closed structure (3). The Grp94 crystal structure (2) may represent an intermediate along this path or non-catalytic conformation. The hydrolysis of ADP causes a further compaction of the protein (4) and the release of nucleotide restarts the conformational cycle.

conformational flexibility and nucleotide dependence map out the basic steps of a nucleotide hydrolysis cycle (Fig. 6).

Molecular dynamics simulations of full-length Hsp90 found an increase in communication efficiency between residues in the NTD and CTD upon the addition of nucleotide (Morra *et al.* 2009). Interestingly, while both ATP- and ADP-bound Hsp90 show residues communicating over a distance of 80 Å, these studies suggest each nucleotide activates a different set of communicating residues between the NTD and CTD as shown in Fig. 7. These different pathways may determine the different conformations of Hsp90 in the presence of ATP or ADP; mutational studies could be used to elucidate each network's role in establishing Hsp90's conformation.

5. The role of co-chaperones in regulating the conformational cycle of Hsp90

In eukaryotes, Hsp90 is often found in large complexes with co-chaperones, some of which are listed in Table 2. Co-chaperones provide a method for the direct recruitment of different client proteins to Hsp90, a means for regulating the Hsp90 conformational and ATP hydrolysis cycle, and subsequently a level of control over client folding. A genomic comparison of Hsp90 co-chaperones shows that many organisms have a unique repertoire of Hsp90 co-chaperones, suggesting that specific co-chaperone combinations can tailor Hsp90's activity to best suit the client requirements for each particular organism (Johnson & Brown, 2009). In this section we discuss well-studied Hsp90 co-chaperones, their influence on Hsp90 conformational dynamics and their role in kinetic regulation.

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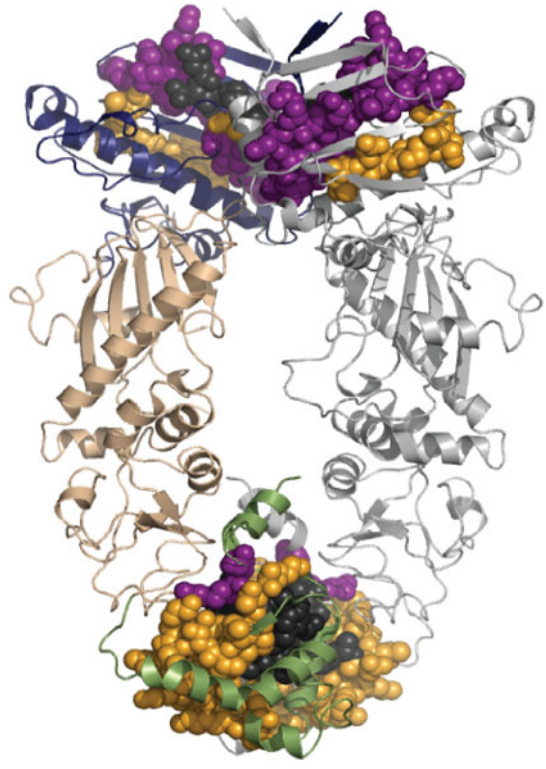


Fig. 7. Molecular dynamics simulations have shown different long-range communication networks between the NTD and CTD in the presence of ATP or ADP. Residues that participate in long-range communication are shown as spheres on the yeast AMPPNP-bound crystal structure. The NTD is shown in blue, the MD in beige and the CTD in green with the second monomer shown in gray. Long-range interactions observed only in the presence of ATP are shown in purple. Interactions seen only in the presence of ADP are shown in orange. Interactions observed with both ATP and ADP are shown in dark gray.

A minimal set of necessary co-chaperones has been established for the progesterone receptor (PR), the GR and the kinase Chk1 providing information about the role of different co-chaperones and about the specific requirements among client proteins. *In vitro*, GR requires only Hsp70 and Hop in addition to Hsp90 for the reconstitution of steroid-binding activity (Arlander *et al.* 2006; Dittmar & Pratt, 1997; Kosano *et al.* 1998). For the *in vitro* reconstitution of PR, Hsp70, Hsp40, Hop and p23 are required in addition to Hsp90 (Kosano *et al.* 1998). The kinase Chk1 also requires Hsp90, Hsp70, Hsp40 and Hop for maximal activation, but unlike PR, the kinase-specific co-chaperone p50 (Cdc37) is also required while p23 is not (Arlander *et al.* 2006). These differences in co-chaperone requirements may provide a mechanism for tuning the Hsp90 response.

The reconstituted systems reveal that specific complexes are formed at different stages throughout the chaperone cycle (Fig. 8). Both Chk1 and PR bind Hsp40 to begin the cycle. Hsp40 delivers the client to Hsp70 and the initial Hsp90 complex forms by client transfer from Hsp70 facilitated by Hop which can simultaneously bind both Hsp70 and Hsp90 to stabilize a ternary complex (Felts *et al.* 2007). In the case of Chk1, this initial complex also includes p50. Hsp70 dissociates to form late-stage complexes. For Chk1, this includes the kinase, Hsp90 and

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Table 2. *Partial list of Hsp90 associated co-chaperones*

Protein	Function
Aha1	Stimulates ATPase activity*
p50	Blocks ATPase activity and is thought to be kinase specific*
Cpr6	TPR-containing and peptidy-prolyl-isomerase (Johnson *et al.* 2007)
FKBP52	TPR-containing and peptidy-prolyl-isomerase (Riggs *et al.* 2003)
GCUNC-45	TPR-containing that blocks progression of cycle (Chadli *et al.* 2006)
Hop	TPR-containing and scaffolds Hsp70-Hsp90 interaction*
Hsp70	recruits clients to Hsp90*
NudC	CHORD-domain containing (Te *et al.* 2007)
p23	Blocks ATPase activity and traps client proteins in complex with Hsp90*
PP5	TPR-containing and phosphatase*
Sse1	Part of Hsp90 complex in budding yeast (Liu *et al.* 1999)
Tah1	TPR containing (Zhao *et al.* 2005)

For a list of additional co-chaperones see http://www.picard.ch/downloads/Hsp90interactors.pdf
*References can be found in the text.

p50. The steroid hormone receptors are in complex with Hsp90 and p23 at this stage. Activated client proteins are then released from the late-stage Hsp90 complex (Felts *et al.* 2007; Hernandez *et al.* 2002; Kosano *et al.* 1998; Smith, 1993).

The diagrams in Fig. 7 detail the minimal systems required for activation, but numerous other co-chaperones have been identified and are believed to play important roles in the regulation of the chaperone cycle *in vivo* (Table 2). For example, the immunophilin FKBP52 is found in complex with Hsp90, GR/PR, and p23 *in vivo*; FKBP52 also increases the hormone-binding ability of GR and is essential for the correct activation of PR *in vivo* (Chadli *et al.* 2008; Riggs *et al.* 2003). To understand the operation of these large Hsp90/client/co-chaperone complexes, the influence of each co-chaperone on Hsp90 needs to be known.

5.1 p23 arrests the ATPase cycle of Hsp90

Since it was first identified in complex with Hsp90 and unactivated PR (Smith *et al.* 1990), p23 has been shown to have enhanced/diminished binding to Hsp90 under ATP/ADP conditions (Grenert *et al.* 1999; Johnson & Toft, 1995; Sullivan *et al.* 1997). Binding of p23 occurs primarily at the NTD with minor MD contacts (Fig. 9) (Ali *et al.* 2006; Martinez-Yamout *et al.* 2006). The complex promotes and stabilizes a catalytically incompetent closed conformation (McLaughlin *et al.* 2002, 2006; Richter *et al.* 2004; Siligardi *et al.* 2004). The affinity of Hsp90 for p23 can be tuned with Hsp90 mutants that either increase or decrease NTD dimerization (Richter *et al.* 2004; Siligardi *et al.* 2004). As expected, truncation of the NTD domain so that dimerization no longer occurs abolishes the binding of p23.

Promoting the closed state of Hsp90 and inhibiting the ATPase activity has the functional consequence of stabilizing client proteins in a complex with Hsp90 and p23. This has been observed for both GR and PR (Dittmar & Pratt, 1997; Kosano *et al.* 1998). p23 may serve as a molecular timer for the activation of client proteins; once clients are activated p23 is released, hydrolysis occurs and client proteins can dissociate. This timing regulation may occur by two mechanisms: (1) slowed hydrolysis of ATP in the presence of p23 or (2) dissociation of p23 driven by the binding of other co-chaperones. There is evidence for both mechanisms. In yeast,

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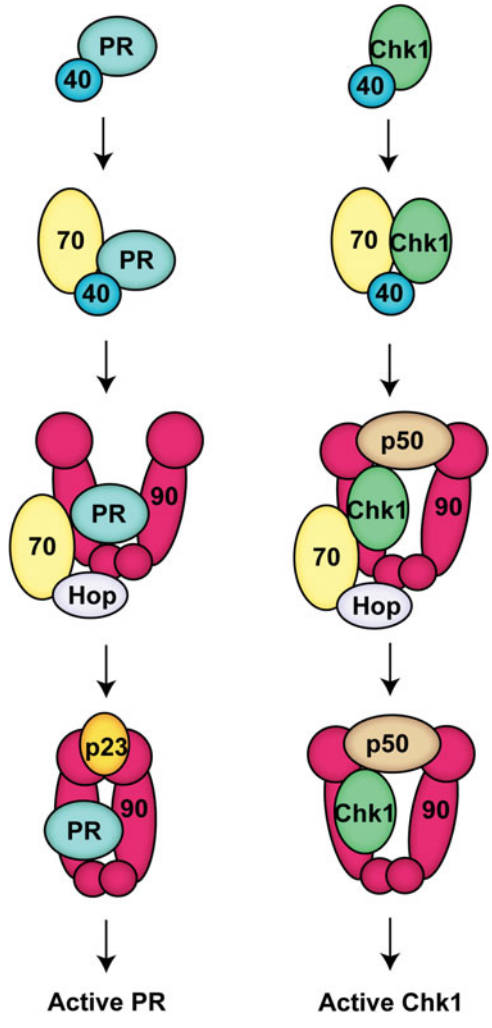


Fig. 8. Proposed chaperone cycles for the activation of PR and the Chk1 kinase. PR or Chk1 is first bound by Hsp40 followed by recruitment to Hsp70. Next, a complex with Hsp90, Hop and Hsp70 forms. This intermediate complex also includes the kinase-specific p50 in the case of Chk1. The mature complex is then formed when Hsp70 and Hop dissociate. The mature complex for PR requires the binding of the additional factor, p23.

Δ8-Hsp90 (increased ATPase activity) hydrolyzes ATP even at saturating levels of bound p23 (Richter *et al.* 2004). Since the wild-type Hsp90 ATPase is strongly inhibited by p23, this indicates a close balance between the natural ATPase activity and p23's inhibition potency. Indeed, the half-life ($T_{1/2}$) of the human p23:Hsp90 complex is 40 s at 37 °C, whereas the $T_{1/2}$ of hydrolysis is 7 min (McLaughlin *et al.* 2006), demonstrating that the kinetics are also comparable. Complete inhibition of human Hsp90's ATPase activity was observed in the presence of p23 but the binding of other co-chaperones can displace p23 and overcome inhibition (McLaughlin *et al.* 2006). The kinetic steps involved in closure, ATP hydrolysis and co-chaperone binding are clearly coupled and likely provide many ways to connect client activation to other cellular processes.

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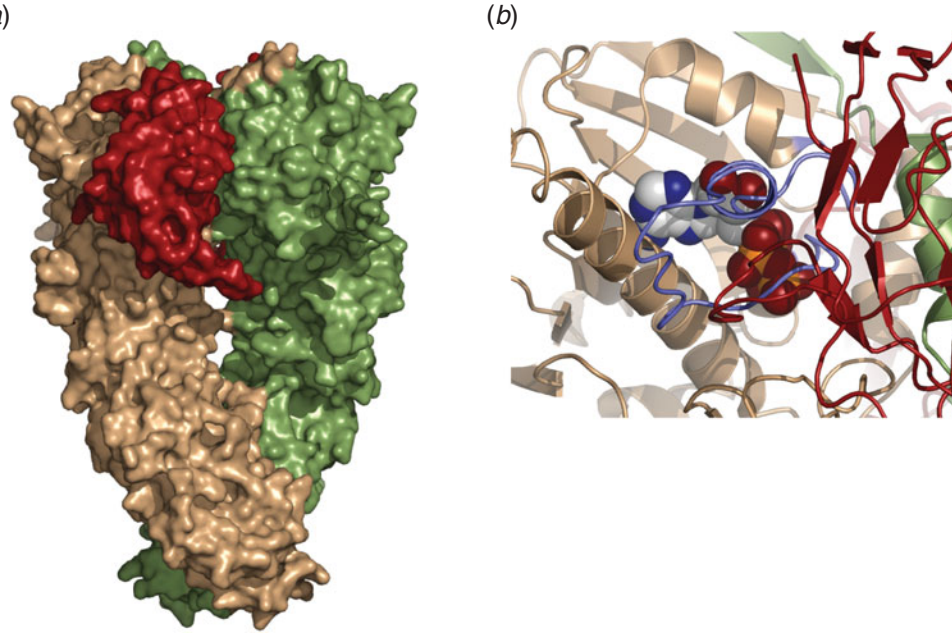


Fig. 9. Crystal structure of the p23:Hsp90 complex. (*a*) Residues 1–134 of yeast p23 were crystallized in complex with the full-length yeast Hsp90 (PDB code, 2CG9). The two monomers of Hsp90 are shown in brown and green whereas p23 is shown in red. (*b*) An expanded view of the ATP-binding pocket shows that the active site lid (shown in light blue) folds over the bound nucleotide when p23 is present. This conformation blocks access in and out of the ATP-binding pocket.

5.2 Hop traps Hsp90 early in the conformational cycle

In contrast to p23, the co-chaperone Hop functions early in the chaperone cycle. Hop is a multidomain TPR protein that facilitates client transfer from Hsp70 (Wegele *et al.* 2006). Although the driving interaction between Hop and Hsp90 is mediated by the C-terminal MEEVD motif of Hsp90, other regions are also involved. The affinity of Hop for Hsp90 decreases with increasing truncations of Hsp90's N-terminus (Richter *et al.* 2003) suggesting that Hop's interaction with Hsp90 spans the entire monomer. In contrast, a biophysical study indicated that the MD/CTD of Hsp90 is sufficient to recapitulate WT Hop binding (Onuoha *et al.* 2008). Further structural studies will be required to reconcile this apparently conflicting evidence.

Hop binds Hsp90 in both the absence and presence of nucleotide although a small decrease in binding in the presence of ATP has been reported (Grenert *et al.* 1999). Like p23, Hop inhibits the ATPase activity of Hsp90, however whereas p23 stabilizes Hsp90 in the closed conformation, SAXS and FRET measurements show that Hop stabilizes Hsp90 in an open state and prevents nucleotide-induced closure (Hessling *et al.* 2009; Onuoha *et al.* 2008). This suggests that Hop and p23 act at different steps in the chaperone cycle by recognizing/stabilizing different conformations of Hsp90.

As with p23, the binding affinity of Hop for Hsp90 can be modulated with conformationally affected Hsp90 mutants. As expected for a model where Hop binds the open state, mutants that promote the closed state show decreased affinity for Hop (Onuoha *et al.* 2008; Richter *et al.*

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2003). The inhibition by Hop can be reversed by the addition of other co-chaperones, such as Cpr6 and PP5, two TPR-containing co-chaperones that compete with Hop for binding to Hsp90 (Prodromou *et al.* 1999). Co-chaperone competition for binding the MEEVD motif may be a general mechanism for controlling the progression through different phases of the Hsp90 conformational cycle. Whether this is a coordinated or stochastic process remains unclear.

5.3 p50, a kinase specific co-chaperone, also traps Hsp90 in an open conformation

The kinase-specific co-chaperone p50 also inhibits the ATPase activity and affects the conformational dynamics of Hsp90, but its mechanism differs from both Hop and p23 (Grammatikakis *et al.* 1999; Lee *et al.* 2002; Stepanova *et al.* 1996). Domain mapping indicates that p50's NTD is critical for binding kinases whereas the middle and CTDs interact with Hsp90 (Grammatikakis *et al.* 1999; Roe *et al.* 2004; Shao *et al.* 2003). Biochemical studies show that p50 binds to Hsp90 in a 1:1 ratio with the interaction occurring largely at the NTD of Hsp90. A crystal structure of the C-terminal region of p50 (residues 148–348) bound to the NTD of Hsp90 shows a dimer for p50 but in this configuration the dimer interface is small and may arise from crystal contacts (Roe *et al.* 2004). Indeed, biochemical studies suggest that p50 binds Hsp90 as a monomer because the K_D for dimerization (80 μM) is higher than the K_D for complex formation (4 μM) (Zhang *et al.* 2004).

The crystal structure (CTD of p50 bound to NTD of Hsp90) indicates a mechanism for the inhibition of Hsp90's ATPase activity. Specifically, residue R167 of p50 points into the nucleotide-binding pocket of Hsp90 and forms a hydrogen bond with the catalytic glutamate of Hsp90 (E33). Nucleotide can still bind, but the catalytic water is no longer coordinated for hydrolysis (Roe *et al.* 2004). The crystal structure also reveals another potential means of inhibition in which p50 blocks the conversion of Hsp90 from the open to the closed state. Specifically, when the p50:Hsp90 dimer is docked onto one NTD of the yeast Hsp90 AMPPNP-bound crystal structure, p50 sterically clashes with the second Hsp90 NTD (Fig. 10). When docked onto the full-length apo crystal form of Hsp90, the p50 dimer fits into the cleft of the Hsp90 dimer potentially stabilizing Hsp90 in an open conformation (Fig. 10*c*).

Two additional lines of evidence support the idea that p50 affects Hsp90 conformational dynamics. First, biochemical studies using the Hsp90 mutants T22I and T101I support the preference of p50 for the open conformation of Hsp90 (Millson *et al.* 2004). Second, for p50 to bind to the full-length apo conformation a structural rearrangement must occur between the NTD and MD of Hsp90 (Fig. 10*c*). Indeed, SAXS measurements of the p50:Hsp90 complex indicate that complex formation causes a contraction in apo Hsp90 (Zhang *et al.* 2004). HX-MS also shows changes in the protection of human Hsp90 in the presence of p50 consistent with a conformational change (Phillips *et al.* 2007).

5.4 Aha1 activates the ATPase of Hsp90

Unlike the other co-chaperones discussed, Aha1 stimulates the ATPase activity of Hsp90 (Lotz *et al.* 2003; Meyer *et al.* 2004; Panaretou *et al.* 2002). Aha1 binds mainly to the MD of Hsp90 and this interaction is mediated by the N-terminal portion of Aha1 (N-Aha1) (Lotz *et al.* 2003; Meyer *et al.* 2004; Siligardi *et al.* 2004). Recent NMR studies suggest that Aha1 also interacts with the NTD of Hsp90 (Retzlaff *et al.* 2010). N-Aha1 has been crystallized in complex with the MD of Hsp90 (Meyer *et al.* 2004) and when aligned with either the closed structure or the open

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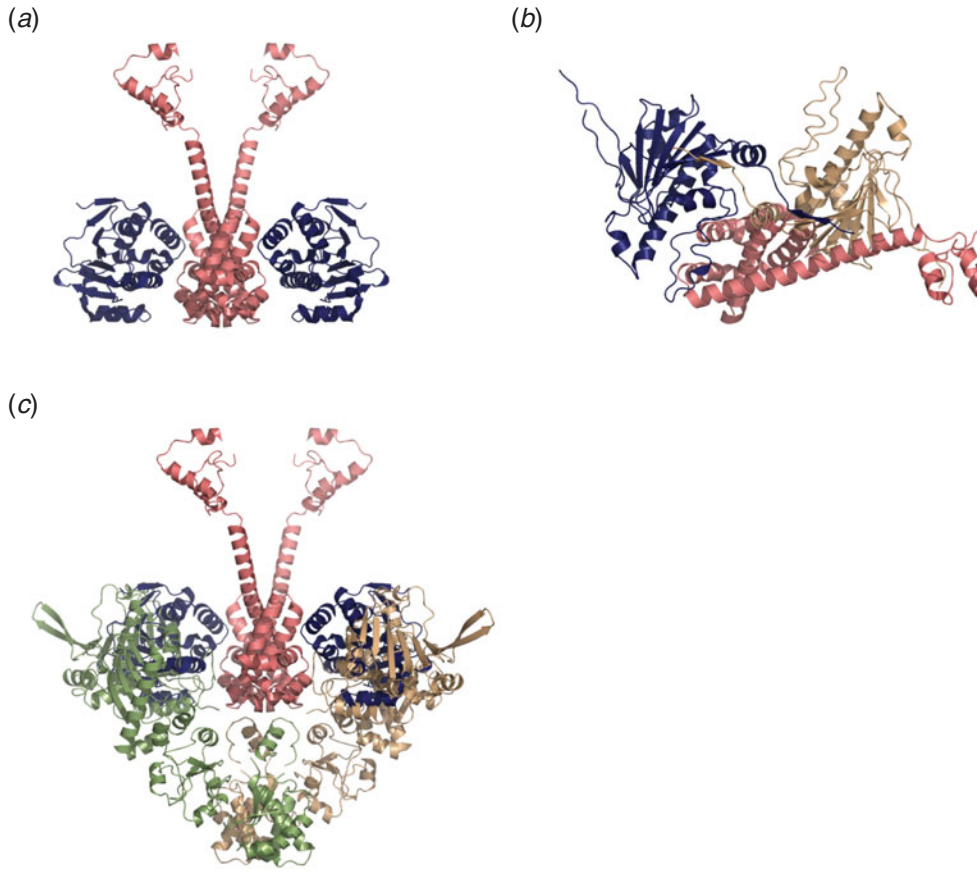


Fig. 10. Crystal structure of p50 bound to Hsp90. (*a*) The C-terminal region of p50 (pink) and the NTD of Hsp90 (blue) form a heterotetramer with p50 forming a dimer between the two NTD domains of Hsp90. (*b*) p50 binding is incompatible with the dimerized NTDs of the Hsp90 closed state. The two NTDs of Hsp90 are shown in blue and beige and they are shown in the configuration seen in the yeast AMPPNP-bound crystal state. Docking of the p50:Hsp90 crystal dimer onto one NTD of the closed state reveals steric clashes between p50 (red) and the second Hsp90 NTD (beige). (*c*) The p50:Hsp90 heterotetramer from A is aligned with the two NTDs from the apo bacterial crystal structure suggesting a mechanism for blocking the closure of Hsp90. One monomer of the apo structure is shown in green and the other in beige. For p50 to bind to this structure, the NTDs of the apo state would have to move as seen by the blue Hsp90 NTDs.

structure, Aha1 binds to Hsp90 at the edge of the dimer cleft approximately 90° from the dimer interface (Fig. 11), and NMR experiments with the full-length proteins suggest that Aha1 binds to both NTDs of the Hsp90 dimer in the closed conformation (Retzlaff *et al.* 2010). Gel filtration and NMR measurements show that Aha1 competes with both Hop and p23 for binding to Hsp90 (Harst *et al.* 2005; Martinez-Yamout *et al.* 2006), indicating that Aha1 can act at multiple stages of the chaperone cycle to disrupt inhibition by other co-chaperones. Aha1 may displace Hop from early complexes and p23 from mature complexes stimulating the ATPase activity and allowing for the continuation of the chaperone cycle.

Aha1 stimulates the ATPase activity of Hsp90 by an asymmetric interaction, and Aha1 bound to one Hsp90 monomer is able to activate both NTDs of the Hsp90 dimer (Retzlaff *et al.* 2010).

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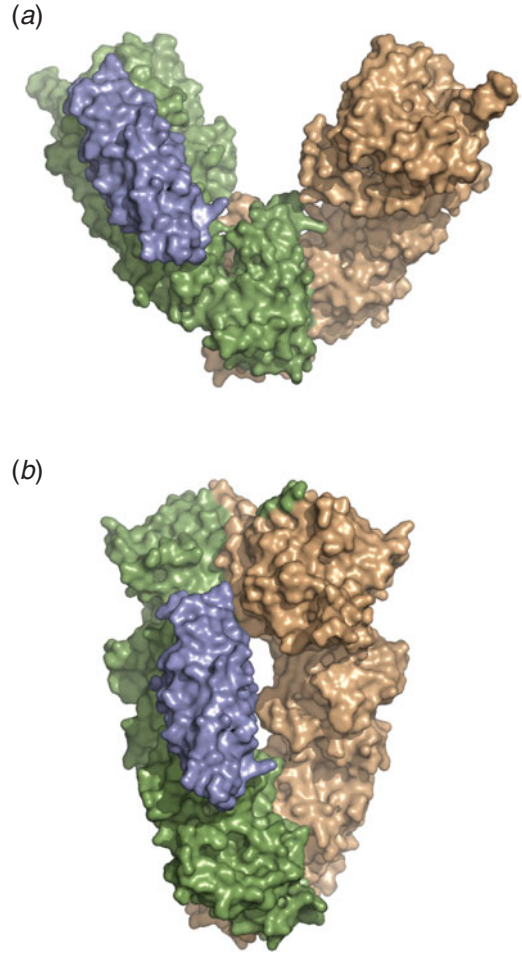


Fig. 11. Aha1 binds Hsp90 on the edge of the cleft. By docking the N-Aha1:MD-Hsp90 complex on the full-length apo (*a*) and ATP (*b*) crystal structures, the approximate orientation of Aha1 can be determined. Aha1 is colored in gray whereas one monomer of Hsp90 is green and the other monomer is beige.

This activation most likely occurs by the interaction of Aha1 with the Hsp90 NM domain optimally positioning the MD catalytic loop for catalysis (Fig. 11). As discussed earlier, in the apo state, the catalytic loop (and the critical R380) is positioned away from the NTD (Fig. 12*b*), whereas in the closed state R380 contacts the γ-phosphate of ATP (Figs 5 and 12*c*). Even though in the Aha1:Hsp90 complex parts of the catalytic loop are disordered, the orientation is much more consistent with the loop conformation found in the closed state (Fig. 12). Furthermore, the R380A mutant, which has a decreased ATPase rate, is not activated by Aha1 (Meyer *et al.* 2003) implying that R380 is critical in the Aha1-mediated activation of Hsp90. Data showing that the Hsp90 F349A mutant, located at the NM interface and potentially involved in interdomain communication, is hypersensitive to Aha1 also support this conclusion (Meyer *et al.* 2003; Siligardi *et al.* 2004).

Although it is clear from these studies that NTD dimerization and rearrangement of the NM domain are important for Aha1's effect on Hsp90, it is not clear as to whether Aha1 simply stabilizes the catalytic conformation once it forms or if binding of Aha1 results in a

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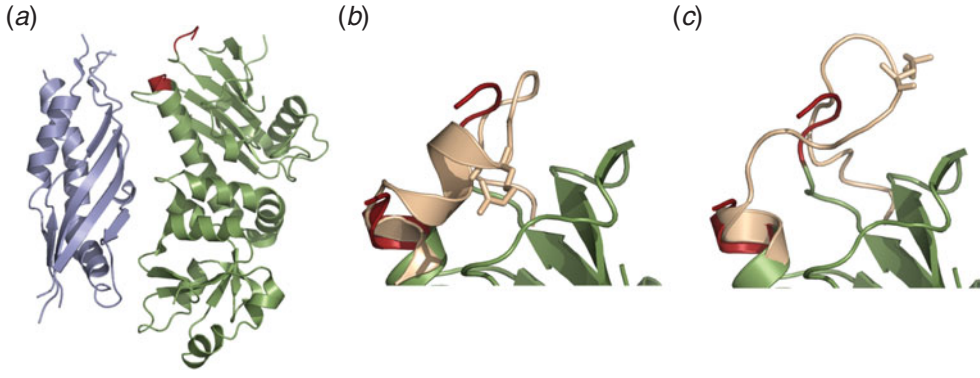


Fig. 12. Aha1 binding to Hsp90 causes a rearrangement in the MD catalytic loop. (*a*) N-Aha1 (gray) was crystallized in complex with the MD of Hsp90 (green). The catalytic loop, shown in red, is partially disordered in the crystal structure. (*b*) The catalytic loop in the apo full-length structure (beige) has more α-helical structure than in the Aha1:Hsp90 complex, and the catalytically required R380 (shown in sticks) forms contacts with the MD in the apo structure. (*c*) In the closed AMPPNP-bound crystal structure the catalytic loop, shown in purple, orients R380 towards the NTD where it interacts with the γ-phosphate of ATP. The loop structure in the Aha1:Hsp90 complex is closely related to the loop structure in the closed state.

conformational change in Hsp90 leading to catalysis. A recent FRET study suggests that Aha1 directly induces structural changes in yeast Hsp90. Aha1-binding results in the rapid acceleration of conformational changes between the NTD and MD similar to the acceleration seen in the lidless Hsp90 mutant, which shows increased NTD dimerization. The FRET results also suggest that Aha1 binding allows Hsp90 to bypass the first conformational intermediate after nucleotide binding leading to an accumulation of the second NTD dimerized intermediate instead (Hessling *et al.* 2009).

6. Client protein binding affects Hsp90 conformation

The conformational dynamics of Hsp90 are likely involved in the folding and activation of client proteins, and changes in Hsp90 likely confer structural changes upon the client protein itself. As another level of control over the Hsp90 cycle, client proteins themselves may also affect the conformation of Hsp90. Early indirect evidence for client proteins altering the conformation of Hsp90 came from *in vitro* studies showing that the ligand-binding domain of GR stimulates the human Hsp90 ATPase rate (McLaughlin *et al.* 2002) suggesting that GR shifts the population of human Hsp90 towards the closed catalytic state. This correlates well with the observation that human Hsp90 has a low basal ATPase rate because it does not significantly populate the closed state. Furthermore, both Hop and p23 inhibit the GR-stimulated rate (McLaughlin *et al.* 2002), as expected based on their effect on conformational dynamics as discussed above.

The clearest structural evidence for client binding influencing the conformation of Hsp90 comes from a 3D negative-stain EM reconstruction of the p50:Hsp90:Cdk4 complex (Vaughan *et al.* 2006). The reconstruction shows an asymmetric complex with two Hsp90 monomers, one p50 monomer and one Cdk4 kinase monomer. The Hsp90 dimer is in a conformation similar to the closed AMPPNP-bound state but the NTDs are not dimerized. One NTD hinges backwards away from the other and p50 binds between the two NTDs. Cdk4 interacts with the outer edge

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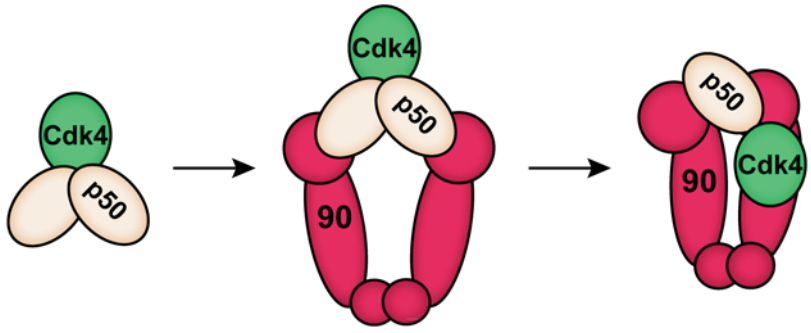


Fig. 13. Proposed model for Hsp90:p50:Cdk4 complex formation. Initially, the kinase Cdk4 binds to a dimer of p50. This complex is then recruited to a dimer of Hsp90 to form a symmetric complex. A conformational rearrangement then occurs and one monomer of p50 is displaced leading to the asymmetric complex observed by EM.

of the complex. The kinase likely contacts the Hsp90 MD with the C-lobe, and the N-lobe contacts one Hsp90 NTD and p50. Previous studies, discussed above, indicate that p50 binds as a 1:1 complex with Hsp90 and this complex is locked in a conformation more closely related to the apo crystal structure. It has been suggested that the kinase initially binds a dimer of p50 and this complex is recruited to Hsp90 forming a symmetric complex. A structural rearrangement then occurs displacing one p50 monomer and leading to the asymmetric complex observed by EM (Fig. 13) (Vaughan *et al.* 2006).

Both GR and kinase clients are actively recruited to Hsp90 by co-chaperones, which adds a layer of complexity to the chaperone/client interactions. Understanding how Hsp90 interacts with just a client is crucial for building more complex models involving the additional involvement of co-chaperones. However, trapping Hsp90 with a mostly unfolded protein presents significant technical challenges. As a solution to this problem, Hsp90/client interactions have been measured with a constitutively unfolded protein Δ131Δ, a well-studied fragment of staphylococcal nuclease. While Δ131Δ is largely unfolded it does not aggregate even at high concentrations. These properties have made it an ideal tool for investigating structural properties of unfolded proteins (Shortle, 2002). Binding of this unfolded protein to Hsp90 drives large-scale conformational changes. Specifically, under apo conditions Hsp90 adapts its conformation by partially closing around Δ131Δ. In the presence of AMPPNP, Δ131Δ binds with increased affinity to Hsp90's fully closed state, accelerates the open/closed transition and stimulates ATP hydrolysis (Street and Agard, in preparation). Further studies will be required to see how Hsp90 responds to different unfolded proteins, and how co-chaperones modulate the Hsp90 response.

A central unanswered question concerns the physical state of Hsp90-bound clients; currently there is conflicting evidence. For example, studies of thermally denatured citrate synthase suggest that Hsp90 interacts in early unfolding or the late stages of folding (Jakob *et al.* 1995). In contrast, NMR studies of a p53 mutant suggest that Hsp90 binds after complete client unfolding (Rudiger *et al.* 2002). Additionally, Δ131Δ has significant residual structure despite being globally unfolded (Alexandrescu *et al.* 1994; Alexandrescu & Shortle, 1994). More studies will be required to determine the folding state of Hsp90-bound clients and which structural features are involved in the binding.

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7. The effect of post-translational modifications on the conformational dynamics of Hsp90

Emerging evidence indicates that post-translational modifications regulate specific client protein interactions with Hsp90. Several modifications have been identified including S-nitrosylation, phosphorylation, ubiqutination and acetylation (Blank *et al.* 2003; Wandinger *et al.* 2008). As discussed below, these modifications likely affect multiple aspects of the Hsp90 conformational cycle.

7.1 S-nitrosylation

S-nitrosylation is the covalent attachment of nitric oxide (NO) to a reactive cysteine. Although the *E. coli* and yeast Hsp90s have no cysteines, human Hsp90 is S-nitrosylated at the CTD (residue 597) by endothelial nitric oxide synthase (eNOS), resulting in reduced ATPase activity (Martinez-Ruiz *et al.* 2005). Given that the site of S-nitrosylation is far from the Hsp90 nucleotide-binding pocket, this suggests an indirect mechanism for the ATPase reduction. Indeed, FRET measurements demonstrate that S-nitrosylation at Cys597 leads to decreased dimerization, suggesting that Hsp90's ATPase activity can be regulated by tuning the monomer/dimer equilibrium. eNOS is an Hsp90 client that is activated by the chaperone's ATPase activity, and so the S-nitrosylation of Hsp90 by eNOS may be a feedback mechanism to limit the accumulation of activated eNOS (Martinez-Ruiz *et al.* 2005).

7.2 Phosphorylation

Phosphorylation of Hsp90 has differing consequences on client interactions depending on the client protein involved and the site of phosphorylation. For example, phosphorylation at S225 and S254 in Hsp90 causes decreased affinity for the aryl hydrocarbon receptor (AhR) (Ogiso *et al.* 2004), whereas phosphorylation at Y300 by c-Src is required for the binding of eNOS (Duval *et al.* 2007). Phosphorylation of Hsp90 can also regulate its cellular abundance. Swe1 phosphorylates Hsp90 at Y24 in a cell-cycle-dependant manner, which consequently causes Hsp90 to be ubiquitinated and degraded in the yeast cytosol (Mollapour *et al.* 2010). When mapped to the closed structure, Y24 sits in the interface between the NTDs in a hydrophobic patch shown to be essential for hydrolysis (Ali *et al.* 2006; Cunningham *et al.* 2008). Indeed, mimicking Y24 phosphorylation by mutagenesis leads to decreased ATPase activity and N-terminal dimerization (Mollapour *et al.* 2010). Interestingly, this modification decreases Hsp90 activation of kinases but does not affect steroid hormone receptor maturation. Together, these data suggest the phosphorylation of Hsp90 at Y24 leads to an altered conformational cycle with reduced closure and ATP hydrolysis, and this change in dynamics affects the activation of specific clients.

7.3 Acetylation

Lysine acetylation of Hsp90 can disrupt co-chaperone and client interactions with Hsp90 (Kekatpure *et al.* 2009; Kovacs *et al.* 2005; Scroggins *et al.* 2007; Yu *et al.* 2002). HDAC6 can de-acetylate Hsp90 (Kekatpure *et al.* 2009) and inhibition or knockdown of HDAC6 leads to hyperacetylation (Yu *et al.* 2002). Hsp90 contains at least two acetylation sites and one site has been mapped to K294 (Hsp82 numbering) at the junction of the NTD and MD (Scroggins *et al.*

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2007). Given this location, it is likely that this modification will affect the NTD/MD orientation, which, as discussed earlier, plays a key role in coordinating the catalytic loop with NTD-bound ATP. More studies are needed to elucidate the conformational consequences of this modification and their relationship to the chaperone cycle and client binding.

Although our knowledge of post-translational modifications as they apply to Hsp90 is still in the early stages, it is clear that they play an important regulatory role. With more site-specific information and biophysical studies, it should be possible to gain a more complete mechanistic picture of post-translational modification-mediated regulation of the essential chaperone cycle of Hsp90.

8. Small molecules also shift the conformation of Hsp90

The conformational dynamics of Hsp90 are essential for proper function and multiple layers of regulation affect the conformation, suggesting that small molecules that affect Hsp90's conformational dynamics may have clinical value. Currently, most Hsp90 inhibitors compete for binding with ATP and were not designed to influence the conformation of Hsp90. There are, however, indications that some inhibitors shift the conformation of Hsp90 to states that are incapable of client protein binding.

As would be expected for small molecules occupying the nucleotide-binding pocket, inhibitor binding causes local changes in the lid of Hsp90 (Immormino *et al.* 2004; Stebbins *et al.* 1997). Binding of the inhibitor GA leads to a lid conformation of Hsp90 (Stebbins *et al.* 1997) similar to the conformation seen in the isolated apo human Hsp90 NTD (Fig. 3*a*, red). This conformation is unlike the lid state seen in the nucleotide-bound complexes where hydrophobic surfaces are accessible for NTD dimerization. The local changes in the NTD induced by the inhibitor most likely correlate with changes in the global structure of the Hsp90 dimer. The GA-bound lid conformation suggests that GA and nucleotide have very different effects on the overall structure of Hsp90.

In vivo, the binding of GA to Hsp90 leads to the destabilization and degradation of client proteins (Mimnaugh *et al.* 1996; Schulte *et al.* 1995; Whitesell *et al.* 1994). In most cases, destabilization is due to the disruption of Hsp90:client complexes as observed for the kinases v-Src and Bcr-Abl (An *et al.* 2000). GA also disrupts the binding of p23 (An *et al.* 2000; Sullivan *et al.* 1997), indicating that GA also inhibits closure. GA increases the presence of Hop and Hsp70 in pull-downs of Hsp90 (An *et al.* 2000; Sullivan *et al.* 1997). Although GA binds to an open conformation of Hsp90, this conformation is distinct from the conformation of apo Hsp90. As measured by SAXS, GA causes compaction of Hsp90 but not closure (Zhang *et al.* 2004). Binding of inhibitors clearly affects the local structure of Hsp90, as HX-MS experiments have shown that DMAG, a derivative of GA and radicicol, an inhibitor similar to GA, causes changes in the protection of amide protons in all three domains of Hsp90 (Phillips *et al.* 2007). Radicicol also increases the protease sensitivity of Hsp90 as compared to the apo protein (Nishiya *et al.* 2009).

Other small molecule inhibitors that function via alternative mechanisms may also cause Hsp90 conformational changes. Novobiocin is a CTD-binding inhibitor (Marcu *et al.* 2000a, b; Soti *et al.* 2002) which, like GA and radicicol, inhibits the formation of Hsp90 complexes with client or p23, but unlike GA it also reduces the affinity of Hsp90 for TPR-containing co-chaperones that bind to the CTD (Allan *et al.* 2006; Yun *et al.* 2004). Epigallocatechin gallate (EGCG) the active ingredient found in green tea also directly binds to Hsp90 and acts as an AhR

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antagonist. As opposed to disrupting the interaction of Hsp90 with AhR, EGCG functions by stabilizing the interaction between Hsp90 and AhR (Palermo *et al.* 2005). A cyclic peptide, Sansalvamide A, appears to bind at the NM domain interface, has no effect on ATPase activity, and yet differentially alters client protein binding *in vitro* and client stabilization *in vivo* (Vasko *et al.* 2010). This demonstrates the potential of non-ATP directed inhibitors to show unique client selectivity, presumably by allosteric modulation of Hsp90 conformational dynamics.

9. Concluding remarks

Structural dynamics play an important role in the function of Hsp90, and understanding the complete conformational ensemble is imperative for defining Hsp90's molecular mechanism. Large conformational changes occur throughout the ATPase hydrolysis cycle, and the conformational state is not rigorously determined by the bound nucleotide. Instead nucleotide binding shifts a conformational equilibrium between states that are also sampled in the absence of nucleotide. *In vivo*, Hsp90 will also sample nucleotide-bound and -unbound states simultaneously due to the weak interaction with ATP. For the cytosolic eukaryotic Hsp90s, co-chaperones regulate the conformational state of Hsp90 by shifting the equilibrium, and the finely tuned regulation is essential for proper chaperone function. Other levels of regulation exist in the form of post-translational modifications such as S-nitrosylation, phosphorylation and acetylation, but the exact nature of these effects remains poorly understood. Other modifications will also require investigation to determine their importance in the regulation of Hsp90. Disrupting the conformational dynamics of Hsp90 provides an attractive target for new therapeutics for the treatment of cancer and potentially vascular disease and Alzheimer's, and inhibitors currently in clinical trials show signs of altering the conformation of Hsp90 and thereby disrupting the binding of client proteins.

Although a much clearer picture of the mechanism of Hsp90 has emerged over the past few years, many outstanding questions require investigation. To begin, the nature of the interaction of client proteins with Hsp90 is largely uncharacterized, and it is essential to understand how client protein binding affects the conformation of Hsp90 and vice versa. This information is key to the understanding of how Hsp90's conformational changes relate to the activation of client proteins. Another open question is the origin of the energy required for the remodeling of client protein. Because the ATPase activity is low and not strongly coupled to the conformational changes that occur, it is unclear from where the force originates to drive the conformational changes in Hsp90 and any consequent changes that occur in the client protein. Also, important differences exist between different homologs and each homolog has a uniquely determined balance between conformational states and transition kinetics. Understanding how the equilibria are established and the functional consequences for shifting the equilibria will provide critical information about the molecular mechanism of Hsp90. Ultimately, a more detailed understanding of Hsp90, its dynamics and interactions will pave the way for the development of more targeted therapeutics.

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