ATP hydrolysis by the Hsp90 molecular chaperone requires a connected set of conformational switches triggered by ATP binding to the N-terminal domain in the Hsp90 dimer. Central to this is a segment of the structure, which closes like a "lid" over bound ATP, promoting N-terminal dimerization and assembly of a competent active site. Hsp90 mutants that influence these conformational switches have strong effects on ATPase activity. ATPase activity is specifically regulated by Hsp90 co-chaperones, which directly influence the conformational switches. Here we have analyzed the effect of Hsp90 mutations on binding (using isothermal titration calorimetry and difference circular dichroism) and ATPase regulation by the co-chaperones Aha1, Sti1 (Hop), and Sba1 (p23). The ability of Sti1 to bind Hsp90 and arrest its ATPase activity was not affected by any of the mutants screened. Sba1 bound in the presence of AMPPNP to wild-type and ATPase hyperactive mutants with similar affinity but only very weakly to hypoactive mutants despite their wild-type ATP affinity. Unexpectedly, in all cases Sba1 bound to Hsp90 with a 1:2 molar stoichiometry. Aha1 binding to mutants was similar to wild-type, but the -fold activation of their ATPase varied substantially between mutants. Analysis of complex formation with co-chaperone mixtures showed Aha1 and p50\textsuperscript{de37} able to bind Hsp90 simultaneously but without direct interaction. Sba1 and p50\textsuperscript{de37} bound independently to Hsp90-AMPPNP but not together. These data indicated that Sba1 and Aha1 regulate Hsp90 by influencing the conformational state of the "ATP lid" and consequent N-terminal dimerization, whereas Sti1 does not.

The molecular chaperone Hsp90 is responsible for the in vivo activation or maturation of specific client proteins (reviewed in Refs. 1–4). Crucial to such activation is the essential ATPase activity of Hsp90 (5), which drives a conformational cycle involving transient association of the N-terminal nucleotide-binding domains within the Hsp90 dimer (6). A variety of studies have shown that Hsp90 is structurally and biochemically related to DNA-gyrase B and MutL (7–10). Structures of co-crystals of GyrB (11) and MutL (12) with the non-hydrolyzable ATP analogue AMPPNP,\textsuperscript{1} identify a segment of the commonly conserved N-terminal nucleotide-binding domain, that acts as an "ATP lid," closing over the mouth of the nucleotide-binding pocket when ATP is present. Hsp90 also possesses a potential lid segment (Gly\textsuperscript{300},Gly\textsuperscript{112} in yeast Hsp90), which is larger than the equivalents in GyrB and MutL, and unlike those has a fully ordered open conformation in the absence of nucleotide. Although the structure of Hsp90 with bound ATP (or AMPPNP) has not yet been directly observed, biochemical and mutagenesis studies (6) suggest that, as with GyrB and MutL, the lid segment in Hsp90 closes over bound ATP facilitating N-terminal dimerization (6, 13) and docking with the middle segment of the chaperone (10) to form an active ATPase conformation.

A number of mutations isolated either through genetic screens (14) or deliberately engineered, have been found to affect the ATPase activity of Hsp90 in ways consistent with the proposed ATP lid mechanism (6). Thus the T101I mutation within the lid segment appears to stabilize the ATP lid in the open conformation seen in crystal structures of the isolated N-terminal domain (9, 15, 16), substantially decreasing ATPase activity. A107N, also within the lid segment, probably stabilizes the closed conformation greatly enhancing ATP turnover and N-terminal dimerization. T22I, which also enhances ATPase activity, probably does not affect lid closure directly but favors consequent association of the N-terminal domains. Mutations in residues outside the N-terminal nucleotide-binding domain, in particular Phe\textsuperscript{349} in the middle segment, have also been found to have significant effects on ATPase activity (10). How the F349A mutation causes a substantial drop in catalytic activity is not certain, but it is likely that Phe\textsuperscript{349} contributes to the interface between the middle segment and the exposed hydrophobic face of the ATP lid in its closed state. The inherent ATPase activity of Hsp90 is also greatly influenced by some of the co-chaperones with which it cooperates in the activation of client proteins in vivo. Aha1 (and the related Hch1) is an activator of the ATPase of Hsp90 able to stimulate the inherent activity of yeast Hsp90 by ~12-fold and human Hsp90\textbeta by ~50-fold (17). Biochemical studies have shown that Aha1 binds to the middle region of Hsp90 (17, 18), and recent structural studies of the Aha1-Hsp90 core complex suggest that

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\textsuperscript{1} The abbreviations used are: AMPPNP, 5'-adenyl-\beta,\gamma-imidodiphosphosphate; ts, temperature-sensitive; ITC, isothermal titration calorimetry; NAha1, N-terminal domain of Aha1.
the co-chaperone promotes a conformational switch in the middle segment catalytic loop (370–390) of Hsp90 that releases the catalytic Arg380 and facilitates its interaction with ATP in the N-terminal nucleotide-binding domain (19). The co-chaperones Sti1 (Hop in mammals) and p50cdc37, both of which are involved in the recruitment of client proteins to the Hsp90 system, are able to arrest the ATPase cycle of Hsp90 to facilitate client protein loading (20, 21). Recent structural studies have shown that p50cdc37 achieves this arrest by binding to surfaces of the Hsp90 N-domain implicated in ATP-dependent N-terminal dimerization and association with the middle segment, fixing the ATP lid in an open conformation and preventing a trans-activating interaction of the N-domains (22). The mechanism of ATPase arrest by Sti1 is not yet fully understood but is also likely to involve an interaction with the N-terminal domains of the Hsp90 dimer (20, 21). Sba1 (p23 in mammals), which binds preferentially to Hsp90 in its ATP-bound state (6, 8, 13, 24), is also able to slow the ATPase cycle (17) presumably by stabilizing the N terminally dimerized conformation. However, as Sba1 binding is dependent on prior binding of ATP, ATPase activity can never be totally inhibited. Where and how Sba1 binds specifically to the N terminally dimerized Hsp90 is still unknown. To gain further insight into the molecular mechanism of Hsp90, we have now analyzed the effect of various Hsp90 mutations on the ability of co-chaperones to bind to Hsp90 and regulate its ATPase activity.

EXPERIMENTAL PROCEDURES

Protein Production and Hsp90 ATPase Activity Assays—Expression and purification of the following His-tagged proteins were described previously, yeast Hsp90, ts-Hsp90 mutants (T22I, T101I, A107N, and F349A), Sba1, Sti1, the C-terminal domain of Sti1 (CSti1), the C-terminal domain of p50 (Cp50), Aha1, and the N-terminal domain of Aha1 (NAha1) (6, 20, 21). Cp50 was cloned from the pET16d-p50 that was a kind gift from Nick Grammatikakis. Purified proteins were dialyzed against 20 mM Tris, pH 7.5, containing 1 mM EDTA and 1 mM dithiothreitol and then concentrated using Vivaspin concentrators (Sartorius) with an appropriate molecular weight cutoff. The protein concentrations were as follows: Hsp90/35 50.4 mg ml⁻¹, T22I/35 63.8 mg ml⁻¹, T101I/35 69.3 mg ml⁻¹, A107N/35 78.4 mg ml⁻¹, F349A/35 53.2 mg ml⁻¹, Sti1/35 63.1 mg ml⁻¹, CSti1/35 25.1 mg ml⁻¹, Sba1/35 30.1 mg ml⁻¹, Cp50/35 41.1 mg ml⁻¹, Aha1/35 49.8 mg ml⁻¹, and NAha1/35 30.1 mg ml⁻¹. The ATPase assays were performed as described previously (5, 17).
Isothermal Titration Calorimetry and K_d Determinations—Heats of interaction were measured on a MSC system (Microcal) with a cell volume of 1.458 ml. For Sti1 interactions, 10 aliquots of 27 μM Hsp90, T22I, T101I, or A107N were injected into 60 μM Sti1, or 43.8 μM F349A was injected into 4 μM Sti1 at 30 °C in 40 mM Tris, pH 8.0, containing 1 mM EDTA and 5 mM NaCl. For Aha1 interactions, 15 aliquots of 20 μM Aha1 were injected into 22 μM Hsp90, T22I, T101I, or A107N, or 128.5 μM Aha1 was injected into 12.85 μM F349A, or 324 μM NAha1 was injected into 32.4 μM Hsp90 at 30 °C in 40 mM Tris, pH 8.0, containing 1 mM EDTA, 5 mM NaCl, 7 mM MgCl₂, and 5 mM AMPPNP. Sba1 experiments without magnesium-AMPPNP were performed by injecting 15 μl aliquots of 150 μM Sba1 into 30 μM Hsp90 or A107N. Heats of dilution were determined in a separate experiment by diluting protein into buffer, and the corrected data were fitted using a nonlinear least square curve-fitting algorithm (Microcal Origin) with three floating variables, stoichiometry, binding constant, and change in enthalpy of interaction. For the Sba1 experiments without magnesium-AMPPNP the stoichiometry was fixed at 0.5:1.0 (Sba1:ts-mutant).

Circular Dichroism and K_d Determinations—CD spectra were recorded on a nitrogen-flushed JASCO J720 spectropolarimeter as described previously (21). Protein concentrations were determined spectroscopically using the following extinction coefficients: ε(Hsp90, T22I, T101I, A107N, and F349A) = 54,050; ε(Sba1) = 28,650; ε(Cp50) = 26,390; and ε(Aha1) = 50,160. The theory and method of difference circular dichroism for investigating protein interactions has been described in detail previously (21).

RESULTS AND DISCUSSION

Sti1 Binding and ATPase Regulation of Hsp90 Mutants—Sti1 is a potent inhibitor of the Hsp90 ATPase activity, but the precise mechanism by which it achieves this is unknown (20, 23). The stoichiometry of the Hsp90-Sti1 interaction as determined by isothermal titration calorimetry (ITC) is most probably 1:1 (Fig. 1), indicating that one Sti1 dimer interacts with one dimeric Hsp90 molecule, which is consistent with previous observations (20). The K_d for the binding of Sti1 to wild-type Hsp90 was determined by ITC as 0.24 μM (Fig. 1A), consistent with previous results (20). K_d values determined with Hsp90 mutants (T22I, K_d = 0.18 μM; T101I, K_d = 0.18 μM; A107N, K_d = 0.41 μM; F349A, K_d = 0.13 μM) suggest that none of the mutations significantly modify the binding of Sti1 (Fig. 1).

Although the binding of Sti1 was unaffected, its ability to inhibit the ATPase activity of the Hsp90 mutants might still be compromised. ATPase assays showed that Sti1 inhibited the ATPase activity of the T22I and A107N mutants to the same degree as wild-type (Fig. 2A). Sti1 inhibition assays with the Hsp90 T101I and F349A mutants were not possible because of their inherently low ATPase activity. Sti1 inhibition of the T101I mutant is normal, whereas the A107N mutation significantly reduces the requirement for Aha1, that the T22I mutant is less responsive to Aha1, and finally that Aha1 effectively suppresses the F349A mutation.
the N-terminal domain (20) but without preventing nucleotide binding (23). That the A107N mutation does not affect Sti1 binding to Hsp90, nor the ability of Sti1 to arrest the ATPase when bound, suggests that the mechanism of ATPase arrest by Sti1 is distinct from that of p50/Cdc37, which binds specifically to the open conformation of the ATP lid in the N-domain and prevents its closure (22). Furthermore, the lack of the effect of the F349A mutation on Sti1 binding also suggests that arrest by Sti1-mediated ATPase arrest does not involve interference with communications between the N-terminal domain and the middle segment of Hsp90. It is likely that structural studies will be required to fully understand the inhibitory function of Sti1.

**Sba1 Binding and ATPase Regulation of Hsp90 Mutants**—In the presence of saturating AMPPNP, the binding of Sba1 to mutants previously shown to favor an association of the N-terminal domains (T22I, Kd = 2.91 ± 0.23 μM; A107N, Kd = 3.69 ± 0.46 μM) was comparable to that with wild-type Hsp90 (Kd = 1.75 ± 0.46 μM) (Fig. 3). As binding to the T22I mutant evolved relatively small heats and therefore gave a less reliable ITC, its affinity for Sba1 was also measured by difference CD, (Kd = 1.50 μM), and found to be similar to that of wild-type Hsp90 (ITC, Kd = 1.75 μM; CD, Kd = 1.16 μM) (Figs. 3, A and B and 4, A–E). Unexpectedly, in all cases the observed stoichiometry for the interaction of Sba1 with Hsp90, in both ITC and CD measurements, was close to 1:2 (Sba1:Hsp90) (Figs. 3 and 4, A, B, H, and I). The Kd for the binding of AMPPNP to the T101I (37 μM) and F349A mutants (56.1 μM) (Fig. 4, E and F) is similar to Hsp90 (37 μM) (15), so that their lower affinity for Sba1 is not because of a defect in ATP binding itself but rather because of an impaired ability to adopt a stable N terminally dimerized conformation on the binding of ATP to which Sba1 then binds. ATPase assays with the F349A and T101I mutants were not conducted because of their inherently low ATPase activities and lack of interaction with Sba1.

The ATP dependence of Sba1/p23 binding to Hsp90 is well known (25, 26) but has not previously been quantified. When AMPPNP was omitted, Sba1 binding to wild-type Hsp90 was 70-fold weaker (Kd = 120 μM in ITC). Tighter binding was observed with the A107N mutant (Kd = 19.2 μM) (Fig. 5), which displays an enhanced N-terminal dimerization (6), but this was still significantly weaker than the affinity for wild-type or A107N Hsp90s in the presence of AMPPNP. These results are fully consistent with previous suggestions that Sba1 (and p23) binding to Hsp90 is not dependent on ATP binding to Hsp90 per se but is dependent on the N-terminal association that ATP binding promotes (13, 15). Most surprising however was the stoichiometry observed for the Sba1-Hsp90 interaction in both ITC and CD measurements, which showed that a single Sba1 molecule binds to an Hsp90 dimer. Monomer-dimer interactions are rare, but not unknown (27, 28), and usually involve a quasi-symmetric bridging interaction in which equivalent sites in the dimer interact with the monomeric ligand but in non-identical ways. In the case of Hsp90 and Sba1 the sites involved in the dimer interact with the monomeric ligand but in non-identical ways. In the case of Hsp90 and Sba1 the sites involved
Aha1 Binding and ATPase Activation of Hsp90 Mutants—
The stress-regulated co-chaperone Aha1 has previously been shown to be a strong activator of the ATPase activity of Hsp90 (17). The $K_d$ for the binding of Aha1 to wild-type Hsp90 measured here (0.65 $\mu$M) (Fig. 6) was consistent with previous estimates (29). Changes in the difference spectra are consistent with changes in the environment of aromatic residues because of molecular interaction.

Consistent with human p23 studies (29),

**FIG. 4.** Near-UV CD analysis of the Hsp90 and Hsp90 $ts$-mutant interactions with AMPPNP and Sba1. A, near UV-difference CD spectra for titration of Sba1 into Hsp90-AMPPNP (50 and 100 $\mu$M, respectively), obtained by subtracting the spectra of the free Sba1 at $n$ molar equivalents, from Hsp90-AMPPNP-Sba1 mixtures at Hsp90:Sba1 molar ratios of $1:n$. The spectrum for the Hsp90-AMPPNP complex is indicated, and curves a–g are from Hsp90:Sba1 mixtures in the molar ratio of 1:$n$ ($n$ = 0.12, 0.25, 0.38, 0.5, 0.78, 1.06, and 1.69). Changes in the difference spectra are consistent with changes in the environment of aromatic residues because of molecular interaction. B, $K_d$ estimation for the binding of Sba1 to Hsp90-AMPPNP. The titration was continued to saturation and yields a $K_d$ of 1.16 $\mu$M, which is consistent with the ITC data ($K_d$ = 1.75 $\mu$M). C, a job plot showing the point of deflection is reached at a molar ratio of Sba1:Hsp90 of 0.5:1 as predicted from ITC experiments (see legend to Fig. 3). D, near UV-difference CD spectra for titration of Sba1 into T22I-AMPPNP (50.4 and 100.8 $\mu$M, respectively), obtained by subtracting the spectra of the free Sba1 at $n$ molar equivalents, from T22I-AMPPNP-Sba1 mixtures at T22I:Sba1 molar ratios of $1:n$. The spectrum for the T22I-AMPPNP complex is indicated, and curves a–h are from T22I:Sba1 mixtures in the molar ratio of 1:$n$ ($n$ = 0.12, 0.25, 0.38, 0.50, 0.75, 1.0, 1.5, and 2.0). Changes in the difference spectra are consistent with changes in the environment of aromatic residues due to molecular interaction. E, $K_d$ estimation for the binding of Sba1 to T22I-AMPPNP. The titration was continued to saturation and yields a $K_d$ of 1.5 $\mu$M, which is consistent with the ITC data ($K_d$ = 2.91 $\mu$M). F, near UV-difference CD spectra for titration of AMPPNP into F349A (145 $\mu$M), obtained by subtracting the spectra of the free AMPPNP at $n$ molar equivalents, from F349A-AMPPNP mixtures at F349A:AMPPNP molar ratios of $1:n$. The spectrum for the F349A-AMPPNP complex is indicated, and curves a–f are from F349A:AMPPNP mixtures in the molar ratio of 1:$n$ ($n$ = 0.50, 1.0, 1.5, 2.0, 3.0, and 5.0). Changes in the difference spectra are consistent with changes in the environment of aromatic residues because of molecular interaction. G, $K_d$ estimation for the AMPPNP interaction with F349A. The titration was continued to saturation and yields a $K_d$ of 56.1 $\mu$M, which is slightly higher than that for Hsp90 (37 $\mu$M). H, near UV-difference CD spectra for titration of Sba1 into F349A-AMPPNP (50.4 and 100.8 $\mu$M, respectively), obtained by subtracting the spectra of the free Sba1 at $n$ molar equivalents, from F349A-AMPPNP-Sba1 mixtures at F349A:Sba1 molar ratios of $1:n$. The spectrum for the F349A-AMPPNP complex is indicated, and curves a–h are from F349A:Sba1 mixtures in the molar ratio of 1:$n$ ($n$ = 0.12, 0.25, 0.38, 0.50, 0.75, 1.0, 1.5, and 2.0). Changes in the difference spectra are consistent with changes in the environment of aromatic residues due to molecular interaction. I, $K_d$ estimation for the binding of Sba1 to F349A-AMPPNP. The titration was continued to saturation and yields a $K_d$ of 22.2 $\mu$M.
The N-terminal domain of Aha1 also bound to Hsp90 (K_d ≈ 120 ± 10.1 μM), showing that the proteins interact very weakly (without magnesium-AMPPNP) indicating that the proteins interact very weakly in the absence of AMP-/H. The interaction in the absence of AMP-

Hsp90. The K_d of this interaction was measured at 19.2 ± 2.5 μM indicating that the binding of Sba1 to the isolated N-terminal domain of Hsp90 is favored by the A107N mutation over that with Hsp90 (K_d = 120 ± 10.1 μM). ITC of Sba1 injected into the N-terminal domain of Hsp90 in the presence of AMP-PPN. The results show that the binding of Sba1 to the isolated N-terminal domain is disrupted thus supporting the idea that N-terminal dimerization requires C-terminal regions of Hsp90.

The N-terminal domain of Hsp90 in the presence of AMP-PPN. The results show that the binding of Sba1 to the isolated N-terminal domain is disrupted thus supporting the idea that N-terminal dimerization requires C-terminal regions of Hsp90.

Although the binding of Aha1 was not affected by the Hsp90 mutations, the degree to which Aha1 was able to stimulate their inherent ATPase activity varied significantly (Fig. 2, C and D). For the T101I Hsp90 mutant, which has a much lower inherent ATPase than the wild-type does, the Aha1-stimulated ATPase activity was significantly lower in absolute value than that with Hsp90 (K_d = 1.35 μM). F349A, K_d = 0.56 μM) (Fig. 6, A–E). The N-terminal domain of Aha1 also bound to Hsp90 (K_d = 1.75 μM) with an affinity similar to the intact Aha1 (Fig. 6, A and F) indicating that NAha1 is primarily responsible for binding to Hsp90. In all cases Aha1 bound to Hsp90 with a 1:1 stoichiometry consistent with the structural studies of the Hsp90-Aha1 core complex.

The two previously characterized ATPase-activating mutations T22I and A107N (6) were both susceptible to stimulation by Aha1, but the combined influence of the stimulatory mutation and co-chaperone binding was relatively weak without any strong synergy. As with the deactivating T101I, the relative lack of effect of the T22I and A107N mutations on Aha1 activation is again consistent with Aha1 acting at a point in the ATPase pathway after lid closure and N-terminal domain association.

In contrast, the effect of Aha1 on the virtually ATPase-dead F349A mutant was dramatic, effectively suppressing the effect of the mutation. Unlike the other residues for which mutants have been assessed, Phe_349 is not in the N-terminal nucleotide-binding domain itself but is part of a hydrophobic patch exposed on the surface of the first α-β-α domain of the middle segment of Hsp90. Modeling of the ATP-bound closed dimeric conformation of Hsp90 suggests that this hydrophobic patch forms a key part of the complex transient interface between the N-terminal domain of Hsp90 and its bound ATP and the middle segment catalytic loop. By analogy with other GHKL ATPases such as Gyrb and MutL, the assembly of this interface is essential to allow correct positioning of the middle segment catalytic residue Arg_308, which interacts with the γ-phosphate of the nucleotide to promote the key catalytic step of the ATPase reaction, hydrolysis of the β-γ phosphodiester bond. Mutation of Phe_349 would be expected to affect this interface, preventing the formation of the catalytically active conformation of Arg_308, with consequently severe impact on ATP hydrolysis, as observed. The ability of Aha1 to suppress the effect of the F389A mutation, suggests that it acts to facilitate formation of the catalytic active conformation of Arg_308.

Insight into the mechanism by which Aha1 achieves this has come from the recent determination of the structure of a complex between the N-terminal domain of Aha1 (equivalent to the whole of Hech1) and the middle segment of Hsp90. The binding of NAha1 elicits a substantial remodeling of the middle segment catalytic loop in Hsp90 (Pro_277-Ile_388) so that the cat-
alytic Arg<sup>308</sup> is released from a retracted conformation observed in the structure of the unliganded middle segment.

Compatibility of Co-chaperones in Complex with Hsp90—
The number of different Hsp90 co-chaperones identified offers the theoretical possibility of a very wide range of different Hsp90-co-chaperone complexes. However it is clear that some co-chaperones only bind to specific conformational states of Hsp90 (8, 13, 20–22, 24), whereas others have common binding sites and are therefore mutually exclusive (30). The protein kinase specific co-chaperone p50<sub>Cdc37</sub> had previously been thought to bind to Hsp90 at a site overlapping but not identical with the tetratricopeptide repeat-domain binding site in the C terminus (30, 31). However subsequent structural studies (22) have shown that p50<sub>Cdc37</sub> binds to the N-terminal nucleotide-binding domain, reinforcing an ATPase-arrested conformation of Hsp90 (21). To gain some further insight into co-chaperone compatibility we have used difference circular dichroism to analyze formation of Hsp90-based complexes in the presence of p50<sub>Cdc37</sub> and other co-chaperones.

When Hsp90, Aha1, and the C-terminal Hsp90-binding region of p50<sub>Cdc37</sub> (Cp50) were simultaneously present, the observed difference CD spectrum could not be simulated by any combination of observed spectra for the components and their pairwise complexes in which one component remained unbound. The observed spectrum was, however, satisfactorily simulated by linear combination of the observed spectra for Hsp90-Aha1 and Hsp90-Cp50 pairwise complexes (Fig. 7, A and B). This is most simply explained by formation of an Hsp90-bridged three-way complex in which Cp50 and Aha1 make no direct mutual interaction. Consistent with this, the order of addition of Cp50 and Aha1 had no affect on the nature of the complex formed (Fig. 7B). The structurally distinct binding sites on Hsp90 for these co-chaperones would allow for such a three-way complex. p50<sub>Cdc37</sub> is responsible for the recruitment of kinases into the Hsp90 complex, whereas deletion of Aha1 can prevent the activation of v-Src protein kinase (17). Thus it is possible that in vivo the ATPase activity of Hsp90 in kinase complexes, which is down-regulated by p50<sub>Cdc37</sub>, may be poised for activation by Aha1 upon displacement of p50<sub>Cdc37</sub>. Interestingly we also observed that Cp50 could form a complex with Hsp90-AMPPNP (Fig. 7C).

We next investigated the ability of Sba1, AMPPNP, and Cp50 to bind simultaneously to Hsp90. As with the Cp50-Hsp90-Aha1 mixture (see above) the observed ΔCD spectrum...
for a mixture of Sba1, AMPPNP, and Hsp90 could not be simulated by any combination of the observed spectra for the components and their pairwise complexes in which one component remained unbound, indicative of the formation of a three-way Hsp90-AMPPNP-Sba1 complex (Fig. 8A). Although the observed spectrum was most closely approximated by a combination of the observed spectra for Hsp90-AMPPNP and Hsp90-Sba1 mixtures, it could not be reproduced exactly, as there is little complex formation between Hsp90 and Sba1 in the absence of nucleotide.

When Cp50 was added to the system, the observed near-UV CD spectrum for the Cp50, Sba1, Hsp90, and AMPPNP mix-
The affects of the F349A mutation, whereas A107N effectively by-
the formation of this catalytic unit, because it can effectively suppress
lid probably by weakening the interactions of the ATP lid with the
loop of the middle domain. Finally F349A destabilizes closer of the ATP
closed state. This idea is supported by the observation that the
seen. Our results suggest that because Aha1 activates the
bind. However, how this ATP lid is triggered remains to be
led to N-terminal dimerization to which Sba1 can then
conformational switch exists that promotes ATP lid closure
influenced by mutations that appear unrelated, such as A107N
and F349A, and regulated by co-chaperones that bind different
regions of Hsp90, such as Aha1, p23/Sba1, and Cp50, but

As with Aha1, NAha1 activates the ATPase activity of
Hsp90, albeit to a lesser degree than the intact protein (17), but nonetheless it stimulates ATP turnover. Our structure did not show whether NAha1 could stabilize N-terminal dimerization, although the bound NAha1 may be close enough to the N-terminal domains that it might interact with them. It is also not unreasonable that the catalytic loop released from the middle domain of Hsp90 may itself interact with the N-terminal domains as well as the bound ATP and that these interactions might help to stabilize N-terminal dimerization. How-
ever, what is apparent is that the rate-limiting step of ATP
turnover by Hsp90 is simply not ATP hydrolysis (33) but ap-
ppears to consist of a complex restructuring of Hsp90 involving
ATP lid closure, interaction of the ATP lid with the middle
domain of Hsp90, a second molecular switch that releases the
catalytic loop, and finally N-terminal dimerization. Because of
this complex restructuring of Hsp90 its ATPase activity can be
influenced by mutations that appear unrelated, such as A107N
and F349A, and regulated by co-chaperones that bind different
regions of Hsp90, such as Aha1, p23/Sba1, and Cp50, but
nonetheless act by affecting the same overall mechanism.
Hsp90 ATPase activity can also be influenced by the disruption of C-terminal dimerization, which drastically reduces the
ATPase activity of Hsp90 (6). This suggests that the full
ATPase activity of Hsp90 is only attained when the two halves
of Hsp90 cooperate by N-terminal dimerization and in so doing
help to stabilize the formation of the catalytically active unit.

In conclusion, Aha1 and Sba1 seem to regulate Hsp90 by
mechanisms directly involving the conformation of the ATP lid,
and therefore N-terminal dimerization, whereas Sti1 inhibition
does not. The results we have presented so far support the ATP
lid model and suggest that the formation of an N-terminally
dimerized catalytically active molecule involves several confor-
mational switches including the interaction of the N-terminal
domains with the middle-domains of Hsp90, especially around
F349, and release of the catalytic loop for interaction with the
bound ATP (Fig. 9).

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