The chaperone hsp90 is capable of binding and hydrolyzing ATP. Using information on a related ATPase, DNA gyrase B, we selected three conserved residues in hsp90's ATP-binding domain for mutation. Two of these mutations eliminate nucleotide binding, while the third retains nucleotide binding but is apparently deficient in ATP hydrolysis. We first analyzed how these mutations affect hsp90's binding to the co-chaperones p23 and Hop, and to the hydrophobic resin, phenyl-Sepharose. These experiments showed that ATP's effects, specifically, increased affinity for p23 and decreased affinity for Hop and phenyl-Sepharose, are brought on by ATP binding alone. We also tested the ability of hsp90 mutants to assist hsp70, hsp40, and Hop in the refolding of denatured firefly luciferase. While hsp90 is capable of participating in this process in a nucleotide-independent manner, the ability to hydrolyze ATP markedly potentiates hsp90's effect. Finally, we assembled progesterone receptor heterocomplexes with hsp70, hsp40, Hop, p23, and wild type or mutant hsp90. While neither ATP binding nor hydrolysis was necessary to bind hsp90 to the receptor, mature complexes containing p23 and capable of hormone binding were only obtained with wild type hsp90.

The 90-kDa heat shock protein (hsp90) is an abundant and highly conserved protein involved in a diverse array of cellular processes. Its fundamental importance is underscored by its presence in all species studied, from *Escherichia coli* to humans, with a remarkable 40% amino acid identity (1, 2). Additionally, the level of hsp90 expressed in various human and murine tissues represents up to 2% of total protein (3), and deletion studies in yeast have shown that hsp90 is essential for viability (4). The common thread in many of its known activities is the chaperoning of substrate proteins to activate their function. This has been most extensively studied with steroid receptors, where hsp90 is required to fold the hormone-binding domain of these receptors into a conformation with high affinity for steroid (see Ref. 5 for a review). In this process, hsp90 acts in protein heterocomplexes with a number of other proteins, including hsp70, hsp40, and the co-chaperones Hop and p23 (6–8). Heterocomplex formation as well as hsp90/co-chaperone interaction are known to be nucleotide-regulated (9–11). Hsp90 also participates in a more general protein folding process with hsp70, hsp40, and Hop which requires nucleotides (9, 12), and it can hold and stabilize denatured proteins for subsequent refolding by hsp70/hsp40 (13) or GroEL/ES (14). On its own, hsp90 can bind proteins (15–19), peptides (18, 19), and hydrophobic resins (11, 20, 21), but the effect of nucleotides on these activities is variable and may depend upon the substrate involved.

Recently, clear evidence in the form of biochemical (22–24) and crystallographic (25) studies has been presented demonstrating nucleotide binding to hsp90. This binding occurs in the NH2-terminal domain of hsp90 at the same site as geldanamycin binding (22, 25, 26). Geldanamycin is a specific inhibitor of hsp90, known to disrupt a number of hsp90-dependent processes, including activation of the oncogenic tyrosine kinase pp60src (27), steroid receptor hormone binding (28, 29) and translocation (30), regulation of the HSF1 heat shock transcription factor (31, 32), Cdc37-mediated stabilization of the cyclin-dependent kinase Cdk4 (33), refolding of denatured firefly luciferase in reticulocyte lysate (12, 34), and ribonucleoprotein complex formation between hepatitis B virus reverse transcriptase and its RNA primer (35). The effect of geldanamycin on such a wide range of experimental systems points to the importance of nucleotides in hsp90 activity.

Two recent studies have shown that mutant hsp90 proteins deficient in either nucleotide binding or hydrolysis are incapable of supporting growth in yeast (36, 37). In this paper, we use similar mutants to first analyze their effects on hsp90's interactions with nucleotide and hydrophobic resins, and with the co-chaperones p23 and Hop. Then, we show how alterations in these individual interactions affect the assembly and function of higher-order protein heterocomplexes involved in protein refolding and steroid receptor hormone binding.

**EXPERIMENTAL PROCEDURES**

**Hsp90 Mutants**—Residues for mutation in hsp90 were selected by a review of *E. coli* DNA gyrase B residues involved in ATP binding and hydrolysis (38, 39). This revealed three residues shown to participate in magnesium binding (Asn46, Ref. 39), adenine ring interaction (Asp73, Ref. 39), and ATP hydrolysis (Glu42, Ref. 38). Corresponding residues in hsp90 proteins were determined by examination of published sequence comparisons between hsp90 and gyrase B (40, 41). The particular residues in chicken hsp90a were found using a published analysis of hsp90 genes from a wide range of species (1). The equivalent residues between *E. coli* gyrase B and chicken hsp90a are, respectively: Asn46 and Asn46; Asp73 and Asp72; Glu42 and Glu45; Asp73 and Asp72. These were then mutated individually to alanine residues through the use of polymerase chain reaction-directed overlap extension as described previously (42) using clone p7.11 containing the coding sequence for chicken hsp90a (22) as the template. These mutant genes were then subcloned into the pET23 vector (Novagen) for overexpression in E. coli BL21(DE3)pLysS cells (Novagen) following the manufacturer's protocols.

**Protein Purification**—Purification of hsp70, Hop, ydj-1, and p23 were performed as described previously (9, 11). For wild type and mutant hsp90 proteins, bacterial pellets containing overexpressed hsp90 were...
lysed by sonication in 10 mM Tris-HCl, 10 mM EDTA, 10 mM thiglyc erol, pH 7.5, containing a protease inhibitor mixture of 0.1 mM leup eptin, 0.1 mg/ml bacitracin, 77 μg/ml aprotinin, 1.5 μM pepstatin, and 1 mM 4-(2-aminomethyl)benzenesulfonfyl fluoride. The soluble lysate fr action was separated by sequential DEAE-cellulose and heparin-agarose column chromatography, followed by Mono Q FPLC (Pharmacia) as described previously (22). Purified protein was dialyzed and stored in either 10 mM Tris-HCl, 50 mM KCl, 0.1 mM EDTA, 1 mM diithiothreitol, 10% glyc erol, pH 7.5, or 10 mM Tris-HCl, 50 mM KCl, 5 mM MgCl₂, 2 mM diithiothreitol, pH 7.5. Protein concentration was determined by scan ning densitometric analysis of Coomassie-stained SDS-PAGE gels containing known standards of hsp90 as determined by amino acid analysis. ATP-Sepharose Binding—ATP-Sepharose binding was typically performed with 5 μg of purified hsp90 in a final volume of 200 μl of incubation buffer (IB: 10 mM Tris-HCl, 50 mM KCl, 5 mM MgCl₂, 2 mM diithiothreitol, pH 7.5) containing 0.01% Nonidet P-40 (Sigma, currently available as IGEPAL CA-630), and 20 μM Na₃MoO₄, with or without 5 μM ATP. To this mixture was added 25 μl of γ-phosphate-linked ATP-Sepharose resin (provided by Timothy Haystead (Ref. 43), commercially available from Upstate Biotechnologies, Inc.) which was washed and pre-equilibrated with IB before use to remove free nucleotides. Samples were then incubated for 20 min at 30°C with frequent mixing to resuspend the resin. After incubation, samples were chilled on ice and the resin was pelleted. Following removal of unbound proteins from the supernatant, resin pellets were washed with 3 x 1 ml of cold IB. Bound proteins were eluted from the resin by boiling 2 min in SDS sample buffer (2% SDS, 5% β-mercaptoethanol) and resolved by SDS-PAGE (44). p23 Binding—p23 binding assays were typically performed in a final volume of 200 μl of IB (see above) containing 0.01% Nonidet P-40, 20 mM Na₃MoO₄, 5 mM ATP, and an ATP regeneration system consisting of 10 mM phosphocreatine and 7 units of creatine phosphokinase (both Sigma). Variations in nucleotide content are described for each experiment in the figure legends. Five-10 μg of purified p23 were used along with either 5 μg of purified hsp90 or 50 μl of cell lysate from E. coli BL21 strains overexpressing hsp90. In cases where cell lysate was used, 50 nM geldanamycin was added at a concentration of 10 μg/ml (18 μM). Where indicated, geldanamycin was added at a concentration of 10 μg/ml (18 μM). Following incubation, the mixtures were chilled on ice and supplemented with a mixture of 5 μM KCl and 5 mM ATP for 30 min to stop ATP regeneration. Monoclonal antibody P222 (47) was conjugated to protein A-Sepharose resin (Pharmacia) for use in PR isolation, using 1 mg of antibody/1 ml of resin. Treated cytosol (1.6 ml) was added to 25 μl of antibody resin, and PR was adsorbed by a 1.5-h incubation at 4°C with constant, gentle mixing. Following adsorption, pellets were washed with 3 x 1 ml of cold IB. Bound proteins were visualized by SDS-PAGE as described above. Hop Binding—Hop (p60) binding assays were performed in a final volume of 200 μl of IB (see above) supplemented with 0.01% Nonidet P-40 and 20 mM Na₃MoO₄, 5 mM ATP, and an ATP regeneration system consisting of 10 mM phosphocreatine and 7 units of creatine phosphokinase (both Sigma). Variations in nucleotide content are described for each experiment in the figure legends. Five-10 μg of purified p23 were used along with either 5 μg of purified hsp90 or 50 μl of cell lysate from E. coli BL21 strains overexpressing hsp90. In cases where cell lysate was used, 50 nM geldanamycin was added at a concentration of 10 μg/ml (18 μM). Following incubation, the mixtures were chilled on ice and supplemented with a mixture of 5 μM KCl and 5 mM ATP for 30 min to stop ATP regeneration. Monoclonal antibody P222 (47) was conjugated to protein A-Sepharose resin (Pharmacia) for use in PR isolation, using 1 mg of antibody/1 ml of resin. Treated cytosol (1.6 ml) was added to 25 μl of antibody resin, and PR was adsorbed by a 1.5-h incubation at 4°C with constant, gentle mixing. Following adsorption, pellets were washed with 3 x 1 ml of cold IB. Bound proteins were visualized by SDS-PAGE as described above. Results

**RESULTS**

**The N50A and D92A Mutations Prevent Hsp90 Binding to γ-Phosphate-linked ATP-Sepharose, while E46A Does Not**—Protein sequence analyses of type II DNA topoisomerases and the MutL family of DNA repair enzymes revealed the presence of three regions in hsp90’s amino-terminal domain containing sequence homology to these proteins (40, 41). In addition, a predicted structure for this domain of hsp90 suggested similarities with the NH₂-terminal domain of the B subunit of DNA gyrase (48). This domain of gyrase B is known to contain the ATP binding and hydrolysis functions of the subunit (49), and its crystal structure has been determined (39). We used studies of gyrase B’s structure and function along with structural and sequence data for hsp90 to find residues in hsp90 which could be involved in ATP binding and hydrolysis which was assayed at each Mg²⁺ ions are necessary for ATP binding to hsp90 (22, 25), providing a possible point for disruption of binding. An asparagine residue (corresponding to Asn50 in chicken hsp90α) interacts with a magnesium ion in the gyrase B crystal structure (39), and the same interaction is believed to be found in hsp90 (25). We changed this residue to alanine, and the corresponding mutant is called N50A. The second residue we altered was an asparagine at position 92. The crystal structures for gyrase B and hsp90 show that this residue hydrogen bonds with the adenine base (25, 39) of ATP. The likelihood of its importance is enhanced by the fact that it is a charged residue
in an otherwise hydrophobic environment (25, 26). Asp92 was changed to an alanine, producing the D92A mutant. Finally, we sought to generate a mutant hsp90 which would bind ATP, but be unable to hydrolyze it. A study of the gyrase B ATPase reaction demonstrated that mutation of a glutamate residue (corresponding to Glu46 in chicken hsp90α) to alanine or glutamine eliminated ATPase activity while still allowing ATP binding (38). We chose to mutate this residue to alanine, resulting in the E46A mutant. A phylogenetic study of hsp90 proteins shows that the three residues we have selected for mutation are conserved from *E. coli* to humans (1), further indicating their importance. Two of the residues described here have recently been mutated in yeast hsp90 (36, 37) and human hsp90 (37). Those studies demonstrated that changing Asp92 (Asp79 in yeast) to asparagine reduces ATP binding to subde-
sicable levels, and that changing Glu46 (Glu33 in yeast) to alanine reduces ATPase activity to <1% of wild type while keeping ATP binding comparable to wild type.

The effect of these mutations on hsp90 binding to ATP-Sepharose is shown in Fig. 1. In addition to wild type hsp90, only the E46A mutant was capable of binding to the resin. However, it appears that E46A does not bind as well as does wild type hsp90 (compare protein loads to bound protein for each). The inclusion of 5 mM free ATP effectively competes away binding by both the wild type and E46A. In contrast, neither N50A nor D92A is capable of binding immobilized ATP.

This is shown by the small amount of bound protein and the absence of an effect of competing free ATP, indicating that binding is nonspecific. Thus, while mutation of Glu46 has been shown to disrupt hydrolysis of ATP in comparable systems (36, 37), it is only partially effective in preventing binding to ATP. This provides us with an ATP-binding hsp90 that should be deficient in hydrolysis for use in our study. The inability of hsp90 with mutations at Asn50 or Asp92 to bind ATP-Sepharose demonstrates that interactions between hsp90 and complexed Mg++ and adenine, respectively, are essential for binding.

These two mutants, then, are used to show the effect of the loss of ATP binding on hsp90 function. Note that these bacterially expressed hsp90 proteins are not as pure as the baculovirus-expressed ones used in our earlier work (22), which is due to comparatively low expression relative to endogenous bacterial proteins. However, these co-purifying proteins do not appear to interfere with the assays performed here, since side by side controls using baculovirus-expressed wild type hsp90 showed results similar to those using its bacterially expressed counterpart (data not shown). The identity of the contaminants is not known, although the largest molecular weight contaminant in the wild type hsp90 preparation appears to be a degradation product of hsp90 that is able to bind ATP-Sepharose (Fig. 1).
The improved efficacy of ATP influenced by even lower concentrations of ATP. We have previously demonstrated that lower nucleotide concentrations may be used with ATP-γS (11), but the concentrations used here are lower still. Also, it is possible that the increased effectiveness of ATP-γS is due to a difference in hsp90's affinity for it. Thus, while the formation of stable hsp90-p23 complexes in vitro may require a high ATP concentration, our results suggest that a lower concentration may still be sufficient to bring about ATP-dependent changes in hsp90 activity.

Hsp90 Has Reduced Affinity for Hop Upon ATP Binding, but Hydrolysis Is Not Necessary to Effect this Change—Hop (hsp organizing protein, also known as p60) is a co-chaperone known to associate with both hsp90 and hsp70 (46). Cell lysates from several tissues show complexes of Hop with hsp70 and hsp90 (46), and Hop has also been shown to form complexes with each individual hsp in vitro (9, 55). Because hsp70 and hsp90 do not bind each other (55), Hop may serve as a mediator to bring these two heat shock proteins together. The importance of this association is seen in steroid receptor complexes, where Hop is essential for obtaining the steroid-binding conformations of both glucocorticoid and progesterone receptors in vitro (8, 56). Hop also accelerates refolding of denatured firefly luciferase by hsp70 and hsp40 when hsp90 is present (9). Hsp90 and hsp70 appear to bind to separate domains of Hop, allowing the trimeric complex to form (55).

Hop binding by the hsp70 and hsp90 chaperones is regulated by nucleotides. Both hsp70 and hsp90 favor Hop binding in vitro.
ATP Binding and Hsp90 Function

ATP Binding, but Not Hydrolysis, Is Necessary to Reduce Hsp90 Affinity for Phenyl-Sepharose—The hydrophobic resin, phenyl-Sepharose, has been suggested as a model representing a hydrophobic protein substrate for hsp90 (21). Hsp90 binding to this resin does not require ATP (11), supporting the use of the model, since hsp90 binding to selected substrate proteins in vitro has also been ATP independent (13, 16, 19, 57, 58). For one of these substrates, MyoD, it has been shown that the site of binding on hsp90 lies in the carboxyl portion of the protein (58). Similarly, our studies with hsp90 fragments have shown that interaction with phenyl-Sepharose occurs outside of the amino-terminal ATP-binding domain. However, if hsp90 is preincubated with ATP, it will show reduced binding to phenyl-Sepharose, while ADP preincubation does not have an effect (11). It is not clear if ATP hydrolysis is necessary for hsp90 to adopt a conformation which shows reduced affinity for phenyl-Sepharose. To answer this question and to confirm the need for ATP to effect a structural change in hsp90, we have tested the E46A and N50A mutants along with wild type hsp90 in this assay, the results of which are shown in Fig. 5.

Hsp90 was preincubated at 30 °C in the presence or absence of ATP and an ATP regeneration system. Following this preincubation, the mixtures were chilled on ice, and phenyl-Sepharose resin was added for a second incubation on ice to allow hsp90 to bind. The resin pellets were washed, and the remaining protein bound was examined. The presence of E46A and N50A mutations in the ATP-binding domain do not prevent hsp90’s binding to phenyl-Sepharose in the absence of nucleotide, as both appear to bind as well as wild type. However, the addition of ATP to the N50A mutant has no effect on the amount of hsp90 bound, while wild type and E46A forms show a marked decrease in binding. Thus, loss of ATP binding prevents the conformational change necessary to reduce hsp90’s affinity for phenyl-Sepharose. The ATP effect seen with E46A indicates that although ATP binding is necessary for this change, hydrolysis is not.

ATP Binding and Hydrolysis Are Necessary for Hsp90 to Fully Assist Hsp70 in Refolding Thermally Denatured Firefly Luciferase—Hsp70 and ydj-1 (a member of the hsp40 family) have been shown in vitro to form a protein folding system capable of refolding thermally denatured firefly luciferase (12). Although hsp90 is not an essential component of this system, it appears to have a stimulatory effect on the process (9, 12). Moreover, the addition of the co-chaperone Hop generates a synergy whereby the combined effect of Hop and hsp90 is greater than the sum of their individual effects (9). The beneficial effect of hsp90 on luciferase refolding is only partially reduced by geldanamycin, an hsp90-specific inhibitor which binds to the same site on hsp90 as does ATP (22, 25, 26), implying there are separate ATP-dependent and ATP-independent functions of hsp90 in this process (12).

To better understand the role of ATP in hsp90-mediated protein folding, the ATPase-deficient mutant E46A and the ATP binding-deficient mutant N50A were compared with wild type hsp90 in a refolding system including hsp70, ydj-1, and Hop. In this assay, firefly luciferase was thermally denatured at 40 °C, and activity was confirmed to be <1% of its original activity. It was then added to a mixture containing ATP, an ATP-regenerating system, hsp70, ydj-1, and Hop, with or without hsp90, and allowed to refold at 25 °C. To specifically remove the ATP-dependent function of hsp90, some reactions also included the inhibitor geldanamycin (GA). Refolding was measured by the return of luciferase activity, shown by the

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2 J. P. Grenert and D. O. Toft, unpublished results.
results from a typical experiment in Fig. 6A. As demonstrated by the accelerated rise in luciferase activity, all three hsp90 forms tested had a positive effect on refolding in the presence of Hop. This effect is not the result of simply increasing total protein concentration of the reaction, as the mixture already contains over 60-fold more bovine serum albumin than it does hsp90. The success of mutant hsp90s in promoting refolding indicates that hsp90 has some ATP-independent chaperoning function.

Fig. 6B shows the fold stimulation of luciferase activity over hsp70/ycd-1/Hop alone. Wild type hsp90 provided the most benefit by far (p < 0.05 by two-way ANOVA over the time course studied, with a comparison of five experiments), with its greatest impact early in the refolding process. As was seen in vivo in yeast (59), hsp90 speeds refolding without significantly changing the final level of luciferase activity. This is shown by the decreasing effect of hsp90 at the later time points. The N50A mutant was consistently superior to E46A in the absence of GA, although this difference was not statistically significant (p = 0.06 by two-way ANOVA, with a comparison of six experiments). Interestingly, the addition of GA produced similar refolding curves in all three hsp90 forms (Fig. 6, A and B). In order to produce such a result, GA must have a different effect on each hsp90 tested. When the differences in activity caused by GA addition are assessed (Fig. 6C), wild type hsp90 loses up to 40% of its activity with GA, while E46A increases activity by about 10–15% and N50A is unaffected. The differences in GA effect on each mutant are statistically significant over the time course studied (p < 0.05 by two-way ANOVA). These results show that ATP binding and hydrolysis are essential for optimal hsp90 participation in refolding with hsp70. Moreover, E46A’s ability to bind ATP without hydrolysis places hsp90 in a state with inhibitory properties relative to that of an hsp90 molecule unable to bind ATP at all, and this inhibition is relieved by GA. Finally, the absence of a GA effect on the N50A mutant indicates that GA acts only by displacement of ATP.

**Hsp90 Must Hydrolyze ATP to Fold the Progesterone Receptor into a Steroid Binding Conformation—Molybdate ions have been known to stabilize the mature (i.e., steroid-binding) form of the progesterone receptor from chicken oviduct cytosols (60, 61). This form of the receptor is not alone, but in a complex including hsp90, p23, and an immunophilin (FKBP52, FKBP51, or Cyp40) (62). It is believed that in the process of forming this complex, hsp90 forms an earlier, intermediate complex with the receptor that contains hsp70 and Hop (63). Recently, techniques have been developed for assembling steroid receptor complexes from purified proteins. For the glucocorticoid receptor, a series of papers by Dittmar et al. (64, 7, 52) has shown that this requires the addition of hsp70, hsp40, hsp90, Hop, and, for stabilization, p23. Our laboratory has assembled PR complexes with the same set of proteins (8).

Although ATP is essential for assembly, it is not clear where in the process hsp90 utilizes ATP, if at all, and whether or not this utilization involves hydrolysis. Hsp90-p23 complex formation from purified proteins requires ATP, but poorly hydrolyzed ATPγS is superior to ATP in this process (Fig. 3A and Ref. 11). On the other hand, hsp90-Hop pairs form in the absence of ATP. Here, we use the E46A and N50A mutants along with wild type hsp90 to assess the need for ATP binding and/or hydrolysis by hsp90 in forming steroid-binding progesterone receptor complexes.

We immunoprecipitated PR from chicken oviduct cytosol and stripped it of bound proteins. Using purified hsp70, ycd-1, Hop, hsp90, and p23, we reassembled PR complexes at 30 °C in the presence of ATP and molybdate. As shown in Fig. 7A, hsp90 does not have to bind ATP in order to form a complex with PR. The amount of hsp90 present in all cases, including in the presence of the inhibitor GA and the N50A mutation, is greater than the nonspecific background binding seen when PR is not included, although the wild type without GA provides the greatest amount of hsp90 co-precipitating with PR. Similarly, loss of ATP hydrolysis does not exclude complex formation, as indicated by the binding seen with the E46A mutant. The type of complex formed, however, is affected by the ability to bind and hydrolyze ATP. Only by using wild type hsp90 in the absence of geldanamycin will the mature, p23-containing complex be created. The other complexes seen all appear to be of the intermediate type, containing hsp70, Hop, and hsp90 (as well as ycd-1). For wild type hsp90, GA inhibition changes the complex formed from the mature type to the intermediate complex, indicated best by the presence or absence of p23. On the other hand, E46A and N50A form this complex regardless of the presence of GA. Thus, the mutants are “self-inhibitory” in that they cannot progress beyond the intermediate complex without ATP binding and hydrolysis.

Each of these complexes was then incubated at 4 °C with radiolabeled progesterone to assess its ability to bind steroid, indicating the adoption of the PR hormone binding conformation, and the corresponding levels of progesterone binding are shown in Fig. 7B. Not surprisingly, wild type hsp90 best supports the steroid-binding conformation of the PR, with most of this activity lost in the presence of GA. The level of hormone binding does not simply correlate with the amount of hsp90 present, since the dramatic loss of progesterone binding seen with the E46A mutant is accompanied by a much smaller decrease in the amount of E46A protein complexing with receptor (Fig. 7A). The mutants hsp90s, without p23 in their complexes, cannot properly fold PR to allow hormone binding. The effect of GA on them is minimal, since the loss of ATP binding or hydrolysis is already sufficient to lock their receptors in a non-progesterone-binding state. Under these conditions, the presence of a p23-containing complex appears to be more important than binding of hsp90 in the generation of a hormone-binding conformation of PR. The assembly of this complex requires ATP binding as well as hydrolysis by hsp90. The absolute requirement for hsp90 in stabilizing the hormone-binding state is shown by the absence of progesterone binding when hsp90 is not included during incubation.

**DISCUSSION**

With recent evidence that hsp90 not only binds ATP (22–25), but also hydrolyzes it (18, 36, 37), it is important to examine...
how the functions of hsp90 are affected by these processes. At least some key functions of hsp90 that are required for yeast viability are dependent upon its ability to bind and hydrolyze ATP (36, 37). Using ATP binding and hydrolysis mutants, we have been able to separate the individual effects of different steps of hsp90’s ATP cycle. Our studies here indicate that in vitro associations between hsp90 and p23, Hop, and phenyl-Sepharose are regulated only by the conformational changes induced by binding of ATP. This is evidenced by hydrolysis-deficient mutant E46A’s ability to respond to ATP as well as, or better than, wild type hsp90 in these three assays. It is interesting that ATP binding supports binding to p23, yet reduces affinity for Hop and phenyl-Sepharose. Thus, hsp90 appears to have unique sets of associated proteins and substrates for each nucleotide state. The different PR complexes reconstituted from reticulocyte lysate depending on the inclusion of GA (which blocks the ATP-bound state of hsp90) seem to support this idea (62). ATP appears to be readily hydrolyzed under the conditions used here. The significance of hydrolysis in p23, Hop, and phenyl-Sepharose binding assays appears to be to simply reset hsp90 to an ADP-bound or a nucleotide-free conformation, thereby making the ATP-bound conformation transient. This explains the enhanced effectiveness of ATPγS or ATP plus molybdate over ATP alone. Although hsp90 association with the co-chaperones p23 and Hop stabilizes its ATP-bound and nucleotide-free conformations, respectively (see above and Ref. 9), it is not clear if these associations alter hydrolysis activity or the binding and exchange of nucleotides. In a recent study using hsp90 mutants comparable to ours, Obermann et al. (37) also showed that the hsp90/p23 interaction required ATP binding, but not hydrolysis. In addition, they found that p23 did not affect the ATPase activity of hsp90 under their conditions. On the other hand, Prodromou et al. (64) have recently shown that the ATPase activity of hsp90 is inhibited by the yeast Hop homolog, Sti1. It will be important to study the functional consequences of these protein-protein interactions in more detail.

The functions of ATP binding and hydrolysis were tested in two multiprotein complex-based systems: the folding of luciferase and the assembly of PR complexes. These systems differ in their requirement for hsp90. While hsp90 is essential for chaperoning PR to its hormone binding state, it is not essential for luciferase folding, although it stimulates this process. However, ATP binding and hydrolysis are both required for optimal effects on luciferase folding and for PR chaperoning, perhaps because both of these processes require the dynamic assembly

![Fig. 6. Luciferase refolding by hsp70, hsp40, Hop, and wild type or mutant hsp90. 100 nM firefly luciferase was thermally denatured at 40 °C to <1% of its original activity. It was then diluted 1:10 in a final volume of 35 μl of Tris buffer (see “Experimental Procedures”) containing 2 mM ATP, an ATP-regeneration system, 550 nM hsp70, 160 nM ydj-1 (an hsp40 protein), and 100 nM Hop, with or without 180 nM hsp90 (calculated as a monomer). Wild type (w.t. hsp90; squares), E46A (circles), or N50A (triangles) hsp90 were used. This mixture was incubated at 25 °C, and refolding was assayed by measurement of luciferase activity in a 5-μl aliquot removed at each time point. Some samples also contained the hsp90 inhibitor GA at a concentration of 17.8 μM. A, typical refolding curves seen with each form of hsp90 are shown. Refolding by hsp70, ydj-1, and Hop only (No hsp90) is shown by the thick line (diamonds). Samples containing GA are represented by dashed lines and open symbols, versus samples without GA, which are drawn with solid lines and closed symbols. B, the fold increase in luciferase activity upon GA addition is shown. C, the percent change in luciferase activity upon GA addition is shown. This was calculated by comparing the activity at each time point with the activity measured in the absence of hsp90 (which was 100% for each sample). A, B, and C are redisplayed from the data of the authors. The inhibition of luciferase activity by GA is shown in red with open symbols.

FIG. 6. Luciferase refolding by hsp70, hsp40, Hop, and wild type or mutant hsp90. 100 nM firefly luciferase was thermally denatured at 40 °C to <1% of its original activity. It was then diluted 1:10 in a final volume of 35 μl of Tris buffer (see “Experimental Procedures”) containing 2 mM ATP, an ATP-regeneration system, 550 nM hsp70, 160 nM ydj-1 (an hsp40 protein), and 100 nM Hop, with or without 180 nM hsp90 (calculated as a monomer). Wild type (w.t. hsp90; squares), E46A (circles), or N50A (triangles) hsp90 were used. This mixture was incubated at 25 °C, and refolding was assayed by measurement of luciferase activity in a 5-μl aliquot removed at each time point. Some samples also contained the hsp90 inhibitor GA at a concentration of 17.8 μM. A, typical refolding curves seen with each form of hsp90 are shown. Refolding by hsp70, ydj-1, and Hop only (No hsp90) is shown by the thick line (diamonds). Samples containing GA are represented by dashed lines and open symbols, versus samples without GA, which are drawn with solid lines and closed symbols. B, the fold increase in luciferase activity upon GA addition is shown. C, the percent change in luciferase activity upon GA addition is shown. This was calculated by comparing the activity at each time point with the activity measured in the absence of hsp90 (which was 100% for each sample). A, B, and C are redisplayed from the data of the authors. The inhibition of luciferase activity by GA is shown in red with open symbols.
and disassembly of complexes (63). Unlike what is seen in the formation of the binary complexes described above, ATP binding alone is not sufficient for maximum refolding efficiency. Indeed, ATP binding without hydrolysis appears to make hsp90 less effective in the luciferase assay than if it were not bound at all.

Our results show that hsp90 has an ATP-independent dimension to its activity. ATP-negative mutants can enhance the in vitro folding of luciferase, although suboptimally, and they can enter PR complexes, but are unable to chaperone the PR to a hormone binding state. These results indicate that hsp90 can bind and release substrate proteins passively and are consistent with earlier studies showing that hsp90 passively maintains unfolded proteins in a non-aggregated state capable of folding (12, 13, 15–19). However, the full activity of hsp90 may be significantly more complex. In the PR system, hsp90 appears to accept the substrate from hsp70 through a process that is facilitated by Hop (8, 55). The dynamics of this process are unknown, but in vitro, it takes several minutes to accomplish. This suggests that the transfer of PR from hsp70 to hsp90 is not simple and may involve time-consuming events such as major conformational changes of chaperones and substrate. It is also possible that this is an iterative process where the substrate is passed back and forth between hsp70 and hsp90 until it assumes a more advanced folding state. Proper coordination of substrate binding and release may only occur when hsp90 can both bind and hydrolyze ATP.

In the luciferase folding system, protein–protein interactions and complexes have not yet been well defined. However, it is likely that this chaperoning process also involves a dynamic hsp70-Hop-hsp90 complex similar to that for PR since the system functions best when all three proteins are present. In addition, this system may be facilitated by passive interactions between denatured luciferase and hsp90 as demonstrated by previous studies which have shown that hsp90 can, to a lesser extent, enhance luciferase folding in the absence of Hop (9, 12).

Although p23 does not appear to have a role in the luciferase folding system, it is clearly important for PR complexes. The binding of hsp90 to p23 requires ATP binding, but not hydrolysis. In contrast, the PR chaperoning system needs both ATP binding and hydrolysis. This suggests that p23 binding is influenced by substrate binding or other proteins in the PR system. It would seem that hsp90 can assume a conformation capable of p23 binding only after the substrate (PR) has progressed to a near-native conformational state, which requires ATP hydrolysis as described above. Indeed, studies by Pratt and co-workers (7) suggest that p23 can bind hsp90 in the absence of added ATP when hsp90 is bound to the glucocorticoid receptor in an advanced stage of folding. Thus, there may be multiple factors that can influence the conformational state of hsp90.

In considering the interaction of substrates with hsp90, one needs to consider the possibility that hsp90 has two substrate-binding sites. Both Young et al. (19) and Scheibel et al. (18) have shown that the passive chaperoning activity of hsp90 can be accomplished by fragments of the protein near the NH2 terminus and also, near the COOH terminus. Thus, protein substrates may be transferred from one binding site to the other. If so, this process may be controlled by ATP binding or hydrolysis.

Another potential complexity can be envisioned by comparing hsp90 to topoisomerase II, which functions as a dimer and contains an ATP-binding domain with similarities to hsp90 (26, 36). Recent studies indicate a complex, sequential pathway for ATP binding and hydrolysis by topoisomerase II which probably relates to multiple conformational states of the protein (65, 66).

The present studies illustrate the roles of ATP binding and hydrolysis in various hsp90/protein interactions. They provide a basis for future studies that are needed to describe more clearly the characteristics of ATP binding and hydrolysis by hsp90 and the resulting conformational and functional states of this protein.

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3 B. D. Johnson and D. O. Toft, unpublished data.
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