The Influence of ATP and p23 on the Conformation of hsp90*

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The chaperoning activity of the heat shock protein hsp90 is directed, in part, by the binding and hydrolysis of ATP and also by association with co-chaperone proteins. One co-chaperone, p23, binds to hsp90 only when hsp90 is in a conformation induced by the binding of ATP. Once formed, the p23-hsp90 complex is very stable upon the removal of ATP and dissipates at 30° with a half-life of about 45 min. This was shown to be due to the high stability of the ATP-induced state of hsp90, not to the rate of p23 dissociation. Further stabilization of this ATP-induced state is achieved by including molybdate or by use of the ATP analogue ATPγS. This conformational state of hsp90 is correlated with the tight binding of ADP resulting from hydrolysis of bound ATP. Both p23 and molybdate enhance and stabilize the nucleotide-bound state of hsp90, and this state is maximized by the presence of both agents. These results can be explained in a model where the binding of ATP induces a conformational transition in hsp90 that traps the nucleotide and is committed to ATP hydrolysis. p23 specifically recognizes this state and may also facilitate subsequent steps in the chaperoning cycle.

hsp90 takes part in a stepwise progression of complexes to chaperone a class of effector proteins to their mature conformation. These hsp90 client proteins include a variety of transcription factors, protein kinases, and cell cycle regulators (1–4). Steroid receptors have been studied extensively for analyzing the chaperoning activity of hsp90, and these studies have revealed the essential proteins and steps that are needed for this process (5–7). To chaperone the progesterone receptor (PR) to a mature form that can bind hormone, hsp90 and the co-chaperone Hop join hsp70 and hsp40 on the PR to form a multiprotein complex. This intermediate complex can progress in the presence of ATP and additional proteins to a mature complex containing PR with hormone binding activity plus hsp90, p23, and one of several immunophilins. This mature complex is transitory; either the receptor binds hormone and activates or the complex dissociates, allowing the process of chaperoning to begin again (5). This dynamic cycling of PR through complexes of chaperones may confer a level of regulation dependent on the fitness and needs of the cell (8).

The conformation and function of hsp90 are regulated, in part, by the binding and hydrolysis of ATP (9–12). A domain near the N terminus of hsp90 is able to bind ATP with a relatively low affinity, resulting in a slow rate of ATP hydrolysis (9, 13–15). ATP binding appears to promote intramolecular interactions near the N terminus of hsp90 (13). It is also required to generate a conformation of hsp90 that can bind the co-chaperone p23 (12, 16, 17). Whether ATP binding influences additional hsp90 interactions or whether the binding of co-chaperones or client proteins alters ATP binding is unclear. It is known that the binding of the co-chaperone Hop to hsp90 inhibits ATP binding and hydrolysis (18, 19). In addition to this ATP binding domain near the N terminus, a second domain that binds nucleotides has been described recently near the C terminus (20–22). These domains appear to influence one another and, thus, the dynamics of the nucleotide regulation of hsp90 might be quite complex.

p23 was first identified as a component of hsp90 complexes with the PR (23). It has since been observed in hsp90 complexes with such diverse proteins as Fes kinase (24), hsf1 (24), telomerase (25), and hepadnavirus polymerase (26). In agreement with biochemical studies (27), a crystal structure for p23 has been resolved showing it to have a globular β-sandwich structure with a flexible tail at the C terminus (28). The role of p23 in the hsp90 chaperoning pathway is still not clear. It has been shown to stabilize hsp90 complexes with PR or GR. Although not strictly required for the interaction of these client proteins with hsp90, p23 dramatically increases the proportion of PR or GR in a mature complex able to bind hormone (29, 30). p23 can be bound to hsp90 in vitro, but hsp90 must first be treated with ATP and sodium molybdate at an elevated temperature to convert it to the appropriate conformation (12). The role of molybdate is uncertain, but it appears to stabilize this conformational state. It has also recently been indicated that p23 may have a role in the turnover or dissociation of GR-hsp90 complexes (31). p23 has the ability to function as a passive chaperone in vitro in that it can prevent the aggregation of denatured proteins (28, 32, 33). In addition, cellular studies indicate that it can modulate the activities of nuclear receptors even after they have been activated and presumed to be dissociated from hsp90 (34, 35).

In this study we examined ATP binding to hsp90 and the effects of sodium molybdate and p23 on this binding. Both of these agents stabilize nucleotide binding at a stage when ATP is tightly bound to hsp90 and committed to hydrolysis.

EXPERIMENTAL PROCEDURES

Proteins—Recombinant human p23 and chicken hsp90α were purified to near homogeneity as described previously (12, 16).

Nucleotide Binding Assay—The monoclonal antibodies J33, which recognizes p23 (23), and D7α, which recognizes hsp90 (23) with or without bound nucleotide, were adsorbed to protein A-Sepharose (Amersham Biosciences). After washing by centrifugation and resuspension to remove excess antibody and equilibrate the resin into binding buffer (20 mM Hepes, pH 7.5, 0.1% Nonidet P-40, 2 mM dithiothreitol), the resin was divided into 30-μl aliquots. These were supplemented with 9

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The abbreviations used are: hsp, heat shock protein; PR, progesterone receptor; GR, glucocorticoid receptor; Mo, sodium molybdate.
µg of hsp90, 3 µg of p23, and, when indicated, 20 mM sodium molybdate (Mo). The ATP label was added last as a 20× stock so that each aliquot contained ~2 µCi of [35S]-PIATP (Blue NEG503H, 3000 Ci/mmole, PerkinElmer Life Sciences) at a final concentration of 0.05 mCi in the 100-µl incubation. The mixture was incubated at 30 °C for 30 min. The basic binding reaction was concluded by isolation of antibody complexes with four 1-ml washes of binding buffer. After resuspension in 1 ml of binding buffer, one-half of the sample was measured for radioactivity to determine the amount of bound nucleotide, whereas the remainder was examined for resin-bound proteins by SDS-PAGE.

**Nucleotide Exchange Assay**—Samples were prepared as described for the basic binding assay. Following incubation for 30 min at 30 °C, the assay tubes were chilled on ice. Then, 5 mM ATP was added, and the incubation continued at 30 °C for the times indicated. The antibody-bound complexes were washed and analyzed as above.

**Dissociation Assay**—Samples were treated as described for the basic binding assay. Following the incubation at 30 °C, the resins were washed twice with 1 ml of binding buffer and re-suspended in 1 ml of binding buffer without or with sodium molybdate or nucleotide as indicated. The incubation continued either on ice or at 30 °C for the times specified. Resin-bound complexes were then isolated and analyzed.

**p23 Binding Assay**—Samples (200 µl) in binding buffer contained anti-p23 antibody bound to protein A resin, 9 µg of hsp90, 5 µg of p23, 5 mM ATP with or without 20 mM sodium molybdate, and any additions as indicated. Typically, samples were incubated at 30 °C for 30 min to form complexes. The hsp90-p23 complexes were either washed and examined by SDS-PAGE or used in exchange or dissociation studies. The hsp90 exchange assay was similar to the nucleotide exchange assay in that a trace amount of [35S]-labeled hsp90, prepared by in vitro transcription and translation, was mixed with 5 µg of hsp90 and 1.5 µg of p23 in the presence of 5 mM ATP and 20 mM Mo. After complexes were formed, 50 µg of hsp90 (pre-incubated with 5 mM ATP at 30 °C for 30 min to put it in the active conformation) was added in an increased volume of 1 ml, and the incubation continued at 0 °C or 30 °C for the times indicated.

**Protease Treatment**—Samples (100 µl) in binding buffer containing 5 µg of hsp90, 5 mM ATP, and either 10 µg of p23 or 20 mM Mo or both were incubated at 30 °C for 30 min and then transferred to ice. Each sample was divided into four aliquots to which chymotrypsin (0.1 µg) was added for 0, 3, 10, or 30 min. Digestion was stopped with the addition of SDS sample buffer. Protein digests were visualized by Coomassie Blue staining after SDS-PAGE. Native hsp90 was quantified using NIH Image version 1.62.

**RESULTS**

**Conditions for Stable Binding of hsp90 to p23**—We had shown previously that hsp90 in the default state has no affinity for p23 but gains the capacity to bind p23 in vitro when it is incubated at elevated temperature in the presence of ATP (12, 36). This appears to be due to a reversible change in the conformation of hsp90. Optimal conditions for this conformational change include 5 mM ATP, 20 mM Mo, and 0.01% Nonidet P-40. It was suggested that Mo functions by replacing the γ-phosphate lost after ATP hydrolysis to maintain hsp90 in a pseudo-ATP-bound state; however, this has not been established. The detergent, Nonidet P-40, stimulates the rate of hsp90 conversion, but its mechanism is unknown. Some additional properties of this interaction are illustrated in Fig. 1. Constant amounts of hsp90 and p23 were incubated under various conditions, and protein complexes were isolated by adsorption to an antibody resin to p23. Upon incubation for 30 min at 30 °C, hsp90 was able to bind to p23 only if ATP was present (lane 3) but not in the absence of nucleotide (lane 1) or in the presence of ADP (lane 2). In the remainder of Fig. 1, the stability of p23-hsp90 complexes was tested under varying conditions using a two-incubation protocol. The resin-bound complexes (lanes 4–8) formed in the presence of ATP and Mo during the first incubation were washed free of the unbound proteins, ATP and Mo. The complexes were then re-suspended in binding buffer with or without additional ATP, ADP, or Mo as indicated and incubated for an additional 30 min at 30 °C. When neither ATP nor Mo was present in this second incubation, there was a reduction of the hsp90-p23 complex by more than 50% (compare lanes 3 and 7). This loss was amplified by the presence of ADP (lane 8), which has been shown to be a potent inhibitor of the ATP-dependent transformation of hsp90 (12). The complex was most stable in the presence of ATP and Mo (lane 4), but Mo alone (lane 5) was nearly as effective. The presence of ATP alone (lane 6) conferred no increased stability over buffer alone (lane 7).

We found previously that the poorly metabolized ATP analogue ATPγS was ~10 times more potent than ATP at promoting hsp90 binding to p23 (12). Even though the concentration of ATPγS used in Fig. 1 (lanes 9–12) was 0.5 mM, the amount of hsp90 complexed was about the same as found with 5 mM ATP and Mo. When ATPγS was used as the nucleotide, hsp90 binding to p23 did not require Mo stabilization (lanes 9 and 11), as shown previously (12). Furthermore, the protein complexes were stably maintained even after washing and subsequent incubation in either the presence (lane 10) or absence (lane 12) of Mo. This is consistent with a function of Mo that relates to ATP hydrolysis. The most striking difference when using ATPγS is that the hsp90-p23 complexes show high stability when incubated in buffer alone (lane 12). This indicates that the hsp90 conformation that is produced by ATPγS treatment is not readily reversible.

Previous studies have shown that the binding of ATP to hsp90 is of low affinity with rapid rates of association and dissociation (14). However, the results in Fig. 1 show that the ATP-dependent conformational state of hsp90 generated in the first incubation dissipates slowly during the second incubation without nucleotide (*e.g.* lanes 5, 10, and 12). The rate at which hsp90 loses its ability to bind p23 after the removal of ATP from the incubation is shown more clearly in Fig. 2. hsp90 was converted to the ATP-induced conformation by incubation at 30 °C for 30 min in the presence of p23 and either 5 mM ATP alone, 20 mM Mo, or 0.5 mM ATPγS. p23 complexes were then isolated on antibody resin, resuspended in buffer without nu-
It is possible that the gradual loss of hsp90-p23 complexes to the p23 antibody resin. When kept on ice, the rate of p23 dissociation from hsp90 was not observed previously (14). We attempted to measure the binding of radiolabeled ATP to hsp90 using an antibody pull-down assay. This non-equilibrium assay would be successful only if the rate of ATP dissociation from hsp90 were relatively slow under the conditions used. This appears to be the case as shown in Fig. 4. An antibody to hsp90 was used to immunoprecipitate complexes with p23 and ATP. Samples containing 1 μl hsp90 and p23 with Mo plus various concentrations of ATP were incubated for 60 min at 30°C and then chilled and assessed for bound ATP. Saturable binding was observed within the range of 2-5 mM ATP, with half-maximal binding occurring at about 0.5–1 mM ATP.

The specificity of ATP binding was tested using 50 μM [α-32P]ATP plus various competitors (Fig. 5). Although this concentration is far below that needed for binding saturation, it provides optimal sensitivity, i.e., a balance between concentration and specific activity. Binding was inhibited by a 10-fold excess (0.5 mM) of ATP and, to a somewhat greater extent, by ADP, whereas GTP, UTP and CTP were ineffective at this concentration. This specificity is similar to that observed previously for nucleotide binding to the N-terminal domain of hsp90 (16). ATP binding was blocked by treatment with geldanamycin but not with cisplatin (data not shown). This, together with the nucleotide specificity, indicates that the binding observed is at the N-terminal domain and not at the ATP binding site described recently (21) at the C terminus that is inhibited by cisplatin.

Both p23 and molybdate influence the binding of ATP as shown in Fig. 6A. In the absence of p23 and Mo (lane 1), there is little ATP binding above the assay background (lanes 5 and 6). ATP binding is enhanced by either p23 or Mo, but both are required to get optimal binding. The binding of ATP is very temperature-dependent, because no binding above background was observed with incubations on ice (results not shown). This suggests that the binding is not a simple association of components but involves conformational or chemical alterations in hsp90 or both. To address this, the bound nucleotide was extracted and analyzed by thin layer chromatography. This showed that essentially all of the [32P]ATP had been hydrolyzed to [32P]ADP (Fig. 6B). When analyzed versus time of incubation at 30°C, only bound ADP was observed at the earliest temperature tested.
time point of 2 min (data not shown). Thus, either ATP is completely converted to ADP upon binding hsp90, or any bound ATP is readily dissociated and lost during the antibody pull-down assay, leaving only the more tightly bound ADP. Note that, even though ADP is the predominant bound nucleotide, one cannot generate the hsp90-p23 complexes using ADP

\[ \text{Mo} \]

(Fig. 1). This apparently requires the process of ATP binding and hydrolysis.

**p23 and Mo Enhance the Extent and Stability of Nucleotide Binding to hsp90**—The influence of p23 on nucleotide binding is shown in the time-course experiment in Fig. 7. The binding observed using 50 μM ATP progresses very slowly without reaching completion at 60 min. p23 has little influence on the rate of nucleotide binding, but it dramatically increases the yield of the complex. The binding of ATP to hsp90 has been shown to be quite rapid, and the rate of nucleotide binding observed in Fig. 7 is too slow to be considered a simple interaction between hsp90 and ATP. The time course resembles that for achieving p23 binding capacity, which involves a conformational change in hsp90 (12).

The preceding results indicate that the binding of ATP leads to a conformational change in hsp90 to a state in which the dissociation of nucleotide is slow. To confirm this, the turnover rate of hsp90-ATP complexes was analyzed (Fig. 8). Complexes of hsp90, \[ \text{[ATP]} \]

and p23 were first formed for 30 min at 30°C. An 80-fold excess of unlabeled ATP was then added for various
times at 30° to measure the loss of bound radiolabeled nucleotide (ADP). The half-time for this exchange reaction was about 50 min in the presence of Mo and 10 min in the absence of Mo. Thus, under optimal conditions (plus Mo and p23), the rate of nucleotide dissociation from hsp90 is very slow, and this provides a likely explanation for the slow loss of hsp90-p23 complex in Fig. 2.

The influence of p23 on the turnover of bound nucleotide is shown in Fig. 8B. p23 has a dramatic effect on the conformation of hsp90 and essentially eliminates the exchange of bound nucleotide. Note that the optimal condition, plus Mo and p23, is more stabilizing in Fig. 8B than in Fig. 8A. For the experiment in Fig. 8B, p23 was in excess of the hsp90 and was free in solution rather than bound to antibody resin. At the completion of the 30° incubation, the samples were chilled, supplemented with p23 where needed, and adsorbed to p23 antibody resin. Thus, this experiment should more accurately illustrate the stabilizing influence of p23.

The effects of p23 on hsp90 conformation were further explored by comparing the sensitivity of hsp90 to chymotrypsin under various conditions (Fig. 9). hsp90 samples were treated with chymotrypsin for 2, 10, or 30 min and then analyzed for the loss of full-length hsp90. In the presence of ATP alone, almost all of the hsp90 was degraded by chymotrypsin within 30 min. A very similar rate of degradation was observed with treatment in the absence of ATP or other additives (results not shown). However, the addition of ATP plus Mo and p23 produced an hsp90 form that was highly resistant to chymotrypsin treatment. Slightly lower degrees of resistance were obtained when either p23 or Mo was deleted from the mixture. Thus, these conditions produce an hsp90 conformation that is very distinct and apparently more compact than the default conformation of hsp90.

FIG. 8. Exchange of bound nucleotide from hsp90. A, the influence of Mo on nucleotide exchange. Samples (100 μl) contained p23 antibody resin, 9 μg of hsp90, 3 μg of p23, and 50 μM [α-32P]ATP with or without 20 mM Mo. After 30 min at 30°C, an additional 1 ml of binding buffer containing 4 mM ATP and 30 μg of p23 with or without 20 mM Mo was added, and the samples were incubated for 0, 1, 3, 10, 30, or 60 min at 30°C. The resin-bound proteins were washed four times to remove unbound nucleotide, and aliquots were measured for radioactivity. B, the influence of p23 on nucleotide exchange. Samples (100 μl) containing 9 μg of hsp90, 20 mM Mo, and 50 μM [α-32P]ATP with or without 3 μg of p23 were incubated for 30 min at 30°C. Then, 1 ml of binding buffer containing 4 mM ATP and 20 mM Mo with or without 30 μg of p23 was added to the samples and incubated at 30°C for 0, 1, 3, 10, 30, or 60 min. The samples were chilled and transferred to p23 antibody resin, and 30 μg of p23 was added to the samples that lacked p23. The incubation continued at 0°C for 90 min, and then the samples were washed free of unbound nucleotide, and aliquots of each sample were measured for radioactivity.

FIG. 9. Sensitivity of hsp90 to chymotrypsin. Samples (100 μl) contained 5 μg of hsp90 and 5 mM ATP plus 10 μg of p23 or 20 mM Mo as indicated. After incubation at 30°C for 30 min, each sample was divided into 4 aliquots on ice and chymotrypsin (0.1 μg) was added for the times indicated at 0°C. Heating a 100°C for 2 min with SDS sample buffer stopped the digestion. The products of proteolysis were resolved by SDS-PAGE. Panel A shows a region of the Coomassie Blue stained gel containing full-length hsp90. Panel B shows the time-dependent loss of full-length hsp90. Relative density units are shown on the y axis.

FIG. 10. A six-step model of the interaction cycle for hsp90 binding to ATP, molybdate, and p23.
The interaction between hsp90 and p23 is not clearly defined, even though it has been studied for several years. Unique features of this interaction are the strict dependence upon ATP binding to hsp90 and the need to have hsp90 in its dimeric state. Also, multiple domains of hsp90 are needed, and p23 binding thus appears to be very sensitive to the conformational state of hsp90 (12, 17). The present study provides some new insights into the relationships between ATP and p23 binding to hsp90.

Our initial interest was the high stability of the hsp90 state that binds p23 upon removal of excess nucleotide. This was particularly evident when using the poorly metabolized ATP analogue ATPγS that generated an hsp90 state that was not readily reversible. This could not be explained by a stable interaction between hsp90 and p23, because this interaction is quite dynamic with a half-life of about 2 min at 30°C. The stability could, however, be explained by the tight binding of nucleotide to hsp90. Such binding was not anticipated, because previous studies had shown that purified hsp90 has a low affinity for ATP accompanied by rapid on and off rates (14). The previous studies had shown that purified hsp90 has a low affinity for ATP (step 1) and forms a very short-lived species that is then readily dissociated. In their model, the cycle is limited by the slow rate of hydrolysis, not by the commitment step or the dissociation of ADP.

Our results are very compatible with the model cited above. Although ATP can bind rapidly to hsp90, our pull-down assay only measures nucleotide that is trapped at the commitment step, which is a much slower process as shown in Fig. 7. The binding of ATPγS results in an accumulation of the committed hsp90 structure that is unable to proceed through the cycle. The result is similar when using ATP plus Mo. However, with ATP alone, hydrolysis and ADP release occur with little or no accumulation of hsp90 in the committed state and a reduced capacity to bind p23.

An important aspect of this study is the use of Mo as a stabilizing agent. We had proposed previously that Mo acts by occupying the binding site for the γ-phosphate after ATP hydrolysis (12). Thus, hsp90 would remain in the conformation induced by ATP binding. Several observations support this mechanism of Mo action. Effects comparable with Mo have been obtained using other compounds that are known to behave as phosphate analogues such as vanadate, tungstate, berrillium fluoride, and aluminum fluoride (12, 37, 38). As shown here, the Mo-stabilized hsp90 actually contains bound ADP, not ATP, and Mo has no effect when hsp90 is bound with ATPγS. However, there is some evidence that Mo interacts with hsp90 near its C terminus, because it can alter the structure of hsp90 in that region (39). It is possible that Mo interacts near the C terminus, perhaps in relation to nucleotide binding in this region, which has recently been shown to occur (20–22). On the other hand, the specificity of nucleotide binding in the present study indicates that this binding is at the site near the N terminus. Also, the N-terminal ATP binding site has been shown to be essential for promoting the p23 binding conformation of hsp90, and this conformation can be obtained with hsp90 fragments that lack the ATP binding site near the C terminus (16, 17).

The model shown in Fig. 10 is adapted from Weikl et al. (14), and it shows five steps in the cycle of ATP binding and hydrolysis plus an additional step to illustrate the action of Mo. The reversible binding of ATP (step 1) induces a conformational change in hsp90 (step 2) that locks ATP into its binding site, committed for hydrolysis (step 3). Under natural conditions, hsp90 would be returned to its initial conformation by the dissociation of the phosphate and, then, ADP (steps 4 and 6). However, we have included steps 4 and 5 to describe our in vitro conditions in the presence of Mo. We propose that ATPγS traps hsp90 between steps 2 and 3 in a state that is committed for hydrolysis but is unable to do so. When using ATP and Mo, hsp90 is trapped at step 4, where the leaving phosphate is replaced by the more tightly binding Mo to prevent ADP dissociation (step 4) and to maintain the committed conformation of hsp90 (hsp90°). Thus, with this proposed mechanism of Mo action, phosphate must dissociate from hsp90 before ADP.

In this model, p23 binding is confined to the committed state of hsp90. This state is probably the same as the “closed” conformation of hsp90 described recently (13) wherein ATP binding promotes dimer interactions near the N terminus of hsp90. Also, a dimer arrangement is required for the binding of p23 to hsp90 (17). This closed, more compact conformation would be consistent with the increased resistance of hsp90 to chymotrypsin shown in our study.

In the hsp90 cycle, p23 appears to bind to and stabilize the committed hsp90. It does not accelerate the generation of this form (step 2), but seems to retard the conformational change in hsp90 that would occur at step 5. A shortcoming of this model is that it does not show the transient nature of the p23-hsp90 interaction. With the nucleotide stably bound in the committed conformation of hsp90, p23 is able to bind and contribute to the stability of the committed conformation. However, this interaction is brief compared with the bound nucleotide, and there may be multiple interactions between p23 and hsp90 while the nucleotide is bound.

When functioning biologically, hsp90 is thought to undergo this cycle only when it is interacting with a substrate or client protein. With steroid receptors, this first involves binding of hsp40 and hsp70 to the receptor and, then, the recruitment of Hop and hsp90 (1, 6, 7). hsp90 in this complex would then interact with ATP and p23, and these interactions help to change the receptor to a state that is able to bind hormone. This in vitro chaperoning of steroid receptors does not require Mo stabilization, although Mo can stabilize receptor complexes (1, 38). p23 promotes the accumulation of “mature” complexes where the receptor is active in hormone binding.

Even in the presence of a client protein, i.e. the progesterone receptor, the progression to a mature complex is slow (7). Apparently, substantial conformational changes occur in hsp90 and also in the PR. In addition to stabilizing the mature complex, p23 may also facilitate the conformational changes in hsp90, the client protein, or both. This may lead to more efficient production of the mature PR complex. This complex is not static, and the PR appears to continuously cycle through the hsp90 pathway (40). By fostering conformational changes, p23 may facilitate steps 5 and 6 as well (Fig. 10), and this might explain the recent observation that p23 can enhance dissociation of receptor from hsp90 that occurs after binding hormone (31).

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