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Assessment of the ATP Binding Properties of Hsp90*

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Hsp90, one of the most prominent proteins in eucaryotic cells under physiological and stress conditions, chaperones protein folding reactions in an ATP-independent way. Surprisingly, ATP binding and ATPase activity of Hsp90 has been reported by several groups. To clarify this important issue, we have reinvestigated the potential ATP binding properties and ATPase activity of highly purified Hsp90 using a number of different techniques.

Hsp90 was compared to the well characterized ATP-binding chaperone Hsc70 and to two control proteins, immunoglobulin G and bovine serum albumin, that are known to not bind ATP. Hsp90 behaved very similarly to the non-ATP-binding proteins and very differently from the ATP-binding protein Hsc70. Like bovine serum albumin and immunoglobulin G, Hsp90 (i) did not bind to immobilized ATP, (ii) could not be specifically photocross-linked with azido-ATP, (iii) failed to exhibit significant changes in intrinsic protein fluorescence upon ATP addition, and (iv) did not bind to three fluorescent ADP analogues. In contrast, Hsc70 strongly bound ATP and ADP, specifically cross-linked with azido-ATP, and exhibited major shifts in fluorescence upon addition of ATP. Finally, reexamination of the amino acid sequence of Hsp90 failed to reveal any significant homologies to known ATP-binding motifs. Taken together, we conclude that highly purified Hsp90 does not bind ATP. Weak ATPase activities associated with Hsp90 preparations may be due to minor impurities or kinases copurifying with Hsp90.

Cells respond to external stresses such as a sudden increase in temperature with the synthesis of a distinct set of proteins called heat shock or stress proteins (Nover, 1991). The predominant classes of stress proteins including GroE, Hsc70,[1] Hsp90, and small Hsps have been implicated in protein folding as molecular chaperones (Morimoto *et al.*, 1994; Jakob and Buchner, 1994; Buchner, 1996). While the precise molecular mechanism of these chaperones is still under extensive investigation, the ATP dependence of chaperone-mediated protein folding is clearly the hallmark of the GroE and Hsc70 class of stress proteins. Both the ATPase activity of these proteins and its influence on assisted protein folding have been analyzed in detail (cf. Morimoto *et al.*, 1994). In contrast, conflicting evidence exists concerning Hsp90's ATP binding properties and ATPase activity. Hsp90 is one of the most abundant proteins in the eucaryotic cell, even at physiological conditions. In complex with other effector proteins such as Hsc70 and prolyl isomerases, Hsp90 has been implicated as a molecular chaperone in the maturation of specific protein substrates such as steroid receptors and kinases *in vivo* (Pratt, 1993; Smith *et al.*, 1993; Jakob and Buchner, 1994; Buchner, 1996). Interestingly, assembly of these complexes with substrate proteins has been found to be ATP-dependent (Pratt, 1993; Smith *et al.*, 1993). However, since Hsc70 is also involved in the formation of these high molecular weight assemblies, it is not yet clear which of the Hsps is responsible for the ATP requirement. More recently, using *in vitro* folding and unfolding assays, it has been demonstrated that Hsp90 may be a general cytosolic chaperone under physiological (Wiech *et al.*, 1992; Shaknovich *et al.*, 1992; Shue and Kohtz, 1994) and heat shock conditions (Jakob *et al.*, 1995a; Schumacher *et al.*, 1994). These chaperone functions as well as interactions of Hsp90 with estrogen receptors (Inano *et al.*, 1994) were found to be ATP-independent. In contrast, binding of ATP to Hsp90 has been reported (Csermely and Kahn, 1991). Binding of ATP was suggested to result in conformational changes of Hsp90 (Csermely *et al.*, 1993), which in turn would affect the interaction with other proteins (Kellermayer and Csermely, 1995). In addition evidence was presented suggesting that some purified Hsp90 preparations exhibit potent peptide-stimulated ATPase activity with high turnover numbers (Nadeau *et al.*, 1992; 1993). However, Hsp90 purified from other species did not show ATPase activity (Wiech *et al.*, 1993; Nardai *et al.*, 1995).

Whether Hsp90 acts in an ATP-dependent or independent way is of crucial importance for understanding the molecular mechanism of this chaperone. Therefore we have examined the ATP binding properties of Hsp90 in detail using several independent experimental approaches. By including known ATP-binding or non-ATP-binding proteins as positive and negative controls, we were able to show that several of the methods employed previously are not suited to unambiguously demonstrate ATP binding. Those methods that turned out to be reliable demonstrate that Hsp90 does not bind ATP.

MATERIALS AND METHODS

Proteins—Hsp90 and Hsc70 from bovine pancreas as well as Hsp90 from yeast and *Escherichia coli* were purified as described previously (Wiech *et al.*, 1993; Jakob *et al.*, 1995a, 1995b). In addition, heparin-Sepharose was used as a final step in the purification of Hsp90 (Olsson *et al.*, 1995). The protein concentrations of bovine Hsp90 and Hsc70 were determined according to Bradford (1976) using bovine serum

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[1] The abbreviations used are: Hsc70/Hsp70, 70-kDa heat shock protein; CS, citrate synthase; GdmCl, guanidinium chloride; BSA, bovine serum albumin; IgG, immunoglobulin G; Fab fragment, antigen-binding fragment of antibodies; PAGE, polyacrylamide gel electrophoresis; MABA-ADP, *N*-8-(4-*N*′-methylanthranylaminobutyl)-8-aminoadenosine diphosphate; MANT-ADP, 3′-*O*-*N*-methyl-anthraniloyl diphosphate; ϵ-ADP, etheno-ADP.

albumin as a standard. Yeast Hsp90 concentrations were obtained using the published extinction coefficient of 0.73 for a 0.1% solution at 280 nm (Jakob *et al.*, 1995a). Mitochondrial citrate synthase (CS) (EC 4.1.3.7), BSA, RNase A, MAK33 IgG, and the respective Fab fragment were obtained from Boehringer Mannheim GmbH.

Chemicals—C8-ATP-agarose was purchased from Sigma, 8-azido-[α-^{32}P]ATP, and [α-^{32}P]ATP were from ICN and ATP was from Boehringer Mannheim GmbH. Polyethyleneimine-cellulose was obtained from Schleicher & Schüll.

Analysis of the Nucleotide Content of Hsp90—Nucleotides were analyzed by reverse phase chromatography with a 2-ml C18 column (Bishop). Detection was at 254 nm. The isocratic elution system consisted of 50 mM potassium phosphate, pH 7.0, for the analysis of AMP/ADP/ATP and 100 mM potassium phosphate, pH 6.8, 10 mM tetrabutylammonium bromide, [N(C_4H_9)$_4$]Br, and 8% (v/v) acetonitrile for the analysis of GMP/GDP/GTP. Typical retention times for the adenosine nucleotides were 3.3 min (ATP), 3.9 min (ADP), and 4.8 min (AMP) at a flow rate of 2 ml/min at room temperature. The retention times for the guanosine nucleotides were 4.7 min (GTP), 3.4 min (GDP), and 2.5 min (GMP) at a flow rate of 1.5 ml/min.

Prior to injection, the protein was denatured to dissociate any bound nucleotides by the addition of 2 μl of 1 M $HClO_4$ to 20 μl of protein solution. The solution was then kept on ice for 1 min, and 28 μl of 2 M potassium acetate was added to reach neutral pH. The sample was then centrifuged at 5000 rpm for 1 min, and 10 μl of the supernatant was analyzed for nucleotide content as described above.

Chaperone Assay—The influence of Hsp90 and Hsc70 on the thermal aggregation process of CS at 43 °C was monitored in the absence or presence of 1 mM MgATP as described previously (Jakob *et al.*, 1995a).

ATP-agarose—1 ml of ATP-agarose was used in a batch binding procedure at 4 °C in Eppendorf tubes. Each batch was equilibrated with buffer A (40 mM HEPES, pH 7.5, containing 20 mM KCl, 5% glycerol and 5 mM $MgCl_2$). Equilibration was performed by resuspending the column material in 2 resin volumes of equilibration buffer, mixing it on a rotating shaker for 10 min, and then centrifuging it at 14,000 rpm. The supernatant was discarded, and the procedure was repeated five times. 200 μg yeast Hsp90 or bovine Hsc70 were mixed with 5 mg/ml IgG carrier protein and made up to 1 ml with buffer A. The protein mixtures were added to the equilibrated ATP-agarose, and these were incubated for 24 h rotating at 4 °C. The samples were then centrifuged at 14,000 rpm for 10 min. The supernatant was retained on ice for further analysis. The ATP-agarose was then washed five times with buffer A. For each wash, the ATP-agarose was incubated in 1 resin volume of buffer A for 10 min on a rotating shaker and then centrifuged at 14,000 rpm. After each centrifugation, the supernatant was retained on ice (wash 1). The agarose pellet was then washed five times as described with high salt buffer (buffer A + 0.5 M KCl), and each time the supernatant was stored on ice at 4 °C (wash 2). Dissociation of specifically bound protein was initiated by the addition of buffer A supplemented with 0.5 M KCl and 5 mM ATP. This procedure was repeated five times. Finally, the ATP-agarose was washed with 2 resin volumes of 7 M urea in buffer A. Samples were analyzed on 10% SDS-PAGE. All gels were silver-stained.

Cross-linking with 8-Azido-[α-^{32}P] ATP—Yeast Hsp90 (5 μg) or BSA (5 μg) were diluted into 40 mM HEPES-KOH, 10 mM $MgCl_2$, 40 μM 8-azido-[α-^{32}P]ATP, pH 7.5, which was supplemented with various concentrations of non-radioactive ATP. The final volume was 20 μl. After a 10-min incubation at room temperature, samples were placed on ice and cross-linking was induced by a 6 min UV radiation (256 nm) at a distance of 4 cm. After addition of 5 μl of 5 × Laemmli buffer (+ 5% β-mercaptoethanol), the samples were incubated for 5 min at 95 °C and applied to a 10% SDS-PAGE. Finally the gel was autoradiographed.

ATPase Activity—ATPase assays were performed as described previously (Kornberg *et al.*, 1978; Knarr *et al.*, 1995). 4–10 μg of purified yeast Hsp90 or RNaseA were incubated at 37 °C with 100 μM unlabeled ATP, 0.1 μM [α-^{32}P]ATP in 40 mM HEPES-KOH, 1 mM $MgCl_2$, pH 7.5. In some experiments with Hsp90, 1 mM $MnCl_2$ or $CaCl_2$ was used instead of $MgCl_2$. Various time points after start of the incubation aliquots of 3 μl were added to 2 μl of nucleotide-EDTA-mix (12 mM ATP, ADP, AMP, EDTA) to terminate the reaction. Thin layer chromatography on polyethyleneimine-cellulose was performed in 0.5 M LiCl and 2 N formic acid. The polyethyleneimine-cellulose sheets were dried, the spots corresponding to ATP and ADP located with UV-light and excised. The radioactivity in each spot was determined by liquid scintillation and expressed as a fraction of the total recovered radioactivity in each lane.

GdmCl-induced Unfolding of Yeast Hsp90—The unfolding transitions of yeast Hsp90 were monitored by measuring the change in intrinsic fluorescence. The measurements were performed in a 1-cm quartz cuvette in a Perkin-Elmer MPF44A luminescence spectrometer at 20 °C. The tryptophan fluorescence was measured at an excitation wavelength of 295 nm and an emission wavelength of 325 nm. The slit widths were set to 5 nm and 10 nm for excitation and emission, respectively. GdmCl-induced unfolding of yeast Hp90 was performed by dilution of the protein into various concentrations of GdmCl in 40 mM HEPES-KOH, pH 7.5, 5 mM $MgCl_2$ (buffer B). The respective protein concentrations are given in the figure legends. The samples were incubated for 20 h at 20 °C to achieve equilibrium. To study the influence of ATP on the stability of yeast Hsp90, GdmCl-induced unfolding was performed as described in the presence and absence of 2 mM ATP.

Influence of ATP on the Fluorescence of Yeast Hsp90, Bovine Hsc70, BSA, and IgG—The respective proteins were diluted into various concentrations of ATP in buffer B. The respective protein concentrations are given in the figure legends. The samples were incubated for 1 min at 20 °C to achieve equilibrium. Subsequently fluorescence measurements were carried out in a 3-mm quartz cuvette as described above. Fluorescence intensities are expressed as the percentage of total protein fluorescence obtained after subtracting the background intensity of the buffer. Changes in tryptophan fluorescence were corrected for the absorption of ATP (inner filter effect) at 295 nm excitation using the formula,

$$F_{corr} = F_{obs} \cdot a/1 - e^{-a} \text{ (with } a = 2.303 \cdot L_T \cdot \epsilon_L) \qquad \text{(Eq. 1)}$$

where F_{corr} is the corrected fluorescence and F_{obs} is the observed fluorescence after subtracting the background light intensity, L_T is the total ATP concentration, and ϵ_L is the absorption coefficient of ATP (Birdsall *et al.*, 1983).

Analysis of the Binding of Labeled ADP to Hsp90—Fluorescence measurements were performed with an SLM Smart 8000 Photon-Counting spectrofluorimeter (SLM Instruments) at 25 °C. Unless otherwise indicated, the buffer was 40 mM HEPES, pH 7.5, 100 mM KCl, and 2 mM $MgCl_2$.

The fluorescent nucleotides were added to 1 ml of buffer at the concentrations indicated. Subsequently, protein was added and mixed manually. All data are volume-corrected averages of 10 readings of 1-s intervals of photon counting.

The nucleotide analogues 3′-*O*-*N*-methyl-anthraniloyl diphosphate (MANT-ADP), *N*-8-(4-*N*′-methylanthranylaminobutyl)-8-aminoadenosine diphosphate (MABA-ADP), and etheno-ADP (ϵ-ADP) were employed to analyze binding of adenosine nucleotides to Hsp90. The concentrations used were 0.4 μM for the nucleotides and up to 0.85 μM for Hsp90. The emission wavelengths of ϵ-ADP, MABA-ADP, and MANT-ADP were 410, 422, and 440 nm, respectively, and the according excitation wavelengths were 300, 340, and 360 nm.

In control experiments binding of MABA-ADP to the molecular chaperone DnaK (Hsc70 from *E. coli*) was determined by fluorescence. In contrast to MANT-ADP, this nucleotide analogue binds with high affinity to DnaK.[2] The conditions used were: 0.4 μM MABA-ADP, 50 mM Tris/HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 2 mM dithioerythritol, 25 °C. The emission wavelength was 422 nm, and the excitation wavelength was 340 nm.

For competition experiments, 1 mM non-labeled ADP was added to the respective protein-ADP analogue incubation reaction and the fluorescence was monitored.

RESULTS

Purified Hsp90 Is Free of Nucleotides—Hsp90 from different sources (bovine, yeast, *E. coli*) was purified by column chromatography to homogeneity (>98%) as determined by densitometry of Coomassie Blue-stained gels. To examine whether purified Hsp90 contains bound nucleotide, we determined the $A_{280/260}$ ratio. UV spectra of Hsp90 gave typically an $A_{280/260}$ ratio of 1.8–1.9, suggesting that the protein samples were essentially nucleotide-free. To confirm this result, we analyzed the nucleotide content of purified yeast Hsp90 by reversed phase high performance liquid chromotagraphy under denaturing conditions. Neither adenosine nor guanosine nucleotides could be detected (data not shown). Together these results demonstrate that, unlike other ATP-dependent chaperones such as GroE

[2] H. Theyssen, H.-P. Schuster, B. Bukau, and J. Reinstein, submitted for publication.

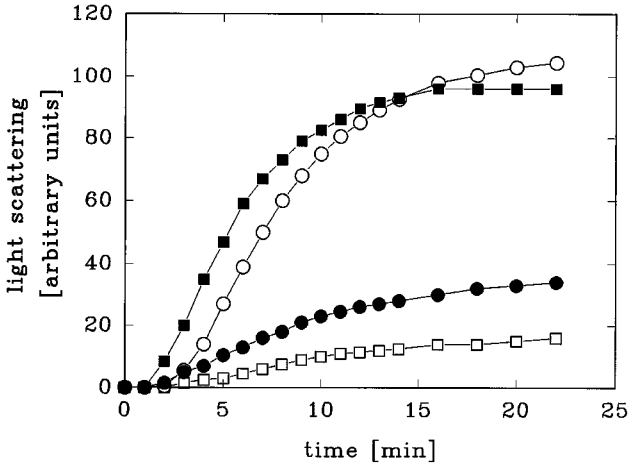

FIG. 1. **Influence of MgATP on the chaperone activity of bovine Hsc70 and bovine Hsp90.** CS (15 μM) was diluted 1:200 into 40 mM HEPES-KOH, pH 7.5, at 43 °C and thermal aggregation was monitored by light scattering. Thermal aggregation of CS in the absence of additional components (○), in the presence of 0.3 μM Hsc70 with (■) and without (●) 1 mM MgATP and in the presence of 0.15 μM Hsp90 with and without 1 mM MgATP (□).

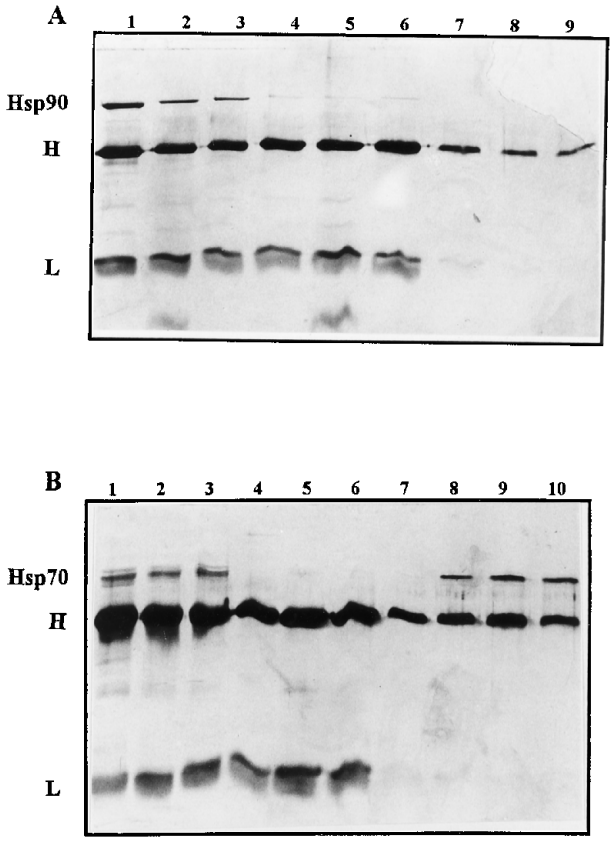

FIG. 2. **Binding of yeast Hsp90 and bovine Hsc70 to ATP-agarose.** 200 μg of yeast Hsp90 (*A*) or bovine Hsc70 (*B*) were mixed with 5 mg of IgG in buffer A containing 5 mM $MgCl_2$, and these were loaded on to 1 ml of ATP-agarose preequilibrated in the same buffer. Samples were analyzed by SDS-PAGE. *A*, *lane 1*, load; *lane 2*, supernatant; *lanes 3* and *4*, wash 1; *lanes 5* and *6*, wash 2; *lanes 7–9*, ATP elution. *B*, *lane 1*, load; *lane 2*, supernatant; *lanes 3* and *4*, wash 1; *lanes 5–7*, wash 2; *lanes 8–10*, ATP elution. Heavy and light IgG chains are denoted by *H* and *L*, respectively.

and Hsc70, purified Hsp90 does not contain bound nucleotides.

The Chaperone Activity of Hsp90 Is ATP-independent—To compare the influence of ATP on the chaperone activity of Hsp90 with that of Hsc70, a known ATP-dependent chaperone, we performed *in vitro* unfolding assays in the presence or absence of ATP with both proteins. As an assay system, we used the thermal unfolding of CS. We had shown previously that Hsp90 suppresses the unspecific aggregation during thermal unfolding and apparently stabilizes CS against inactivation by binding to early unfolding intermediates (Jakob *et al.*, 1995a). As shown in Fig. 1, 1 mM MgATP has no influence on the suppression of CS aggregation by Hsp90. In contrast, suppression of aggregation by Hsc70 is strongly influenced by ATP. This suggests fundamentally different mechanisms of action. In the absence of ATP, Hsc70 effectively prevents aggregation by binding the nonnative protein. In the presence of ATP, however, the protective effect of Hsc70 is lost possibly due to the rapid release and subsequent aggregation of bound protein. Similar effects were observed previously for the chaperone GroE under heat shock conditions (Höll-Neugebauer *et al.*, 1992).

Hsp90 Does Not Bind to ATP-agarose—Binding to immobilized ATP has been taken as evidence for the association of Hsp90 with nucleotides (Csermely and Kahn, 1991). We readdressed the question whether purified Hsp90 binds to immobilized ATP by incubating Hsp90 with ATP-agarose. Initial experiments showed that the addition of the carrier protein IgG was necessary to prevent the unspecific interaction of Hsp90 with the agarose matrix. IgG was shown not to influence the specific binding of the control protein Hsc70 (Fig. 2). To detect specific binding of proteins to ATP-agarose, elution of the bound proteins was induced by competing with free ATP after extensive salt washes. These experiments confirmed that Hsc70 binds to immobilized ATP and can be specifically eluted by the addition of free ATP. However, specific binding of Hsp90 to ATP-agarose could not be detected. The total amount of Hsp90 applied was found in the flow-through and the wash fractions. Addition of divalent cations (Mg^{2+}, Ca^{2+}, and Mn^{2+}) to the incubation buffer did not change the results (data not shown). Therefore, we conclude that Hsp90 does not bind tightly to immobilized ATP.

Azido-ATP Cross-linking to Hsp90 Is Nonspecific—Previously, cross-linking of radiolabeled azido-ATP to Hsp90 has been detected (Csermely and Kahn, 1991; Shaknovich *et al.*, 1992). To investigate whether this represents specific interactions, we compared the cross-linking behavior of Hsp90 with that of the known ATP-binding protein Hsc70 and the non-ATP-binding protein BSA. Competition with cold ATP was used to check the specificity of the cross-linking reaction.

As shown in Fig. 3, cross-linking of azido-ATP to Hsp90 was observed under the conditions used. However, prior to cross-linking we could not compete for azido-ATP binding by addition of a large excess of cold ATP. This shows that azido-ATP and ATP bind to different sites on Hsp90, or that azido-ATP binds with a much higher affinity than normal ATP, or that azido-ATP simply cross-links nonspecifically with Hsp90. Thus the photocross-linking of azido-ATP to Hsp90 cannot be taken as a reliable indicator of ATP binding. This view was reinforced by the finding that BSA (Fig. 3) and Fab fragment (data not shown) photocross-linked to azido-ATP in a manner similar to Hsp90. In contrast, the azido-ATP labeling observed with Hsc70 could be competed with micromolar concentrations of cold ATP.

Since it is known that long-lived reaction intermediates form upon the UV-activation of azido-ATP (Todd *et al.*, 1995), the photocross-linking of azido-ATP with Hsp90 needs to be interpreted with extreme caution.

ATP Does Not Stabilize Hsp90—Substrate or cofactor binding leads to an often dramatic increase in the stabilization

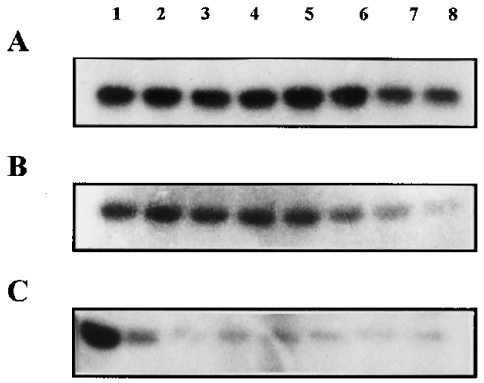

FIG. 3. **Cross-linking of Hsp90, BSA and Hsc70 with 8-azido-[α-^{32}P]ATP.** 5 μg of Hsp90 (*A*), 5 μg of BSA (*B*), or 5 μg Hsc70 (*C*) were incubated with 8-azido-[α-^{32}P]ATP in the presence of increasing concentrations of non-labeled ATP. *Lanes 1–8* represent cross-linking in the presence of 0, 0.05, 0.25, 0.5, 1, 3, 5, and 10 mM non-labeled ATP.

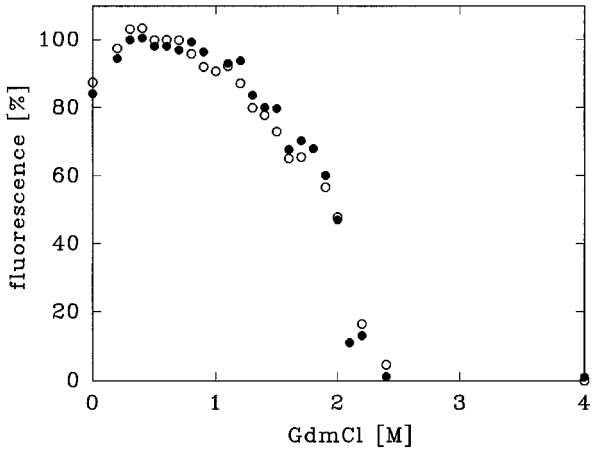

FIG. 4. **Influence of MgATP on GdmCl-induced unfolding of yeast Hsp90.** GdmCl-induced unfolding of yeast Hsp90 (50 μg/ml) was performed in the presence (●) or absence (○) of 2 mM ATP in buffer B. To monitor unfolding of yeast Hsp90, the fluorescence signal at 325 nm was recorded.

energy of the respective protein. This was shown recently for the chaperone DnaK, where binding of MgATP significantly stabilized the protein as determined by monitoring GdmCl-induced unfolding transitions (Palleros *et al.*, 1993). Using this assay system, we analyzed whether the addition of MgATP exhibits similar effects on the stability of Hsp90. In the absence of MgATP an unfolding transition identical to that observed previously (Jakob *et al.*, 1995b) was obtained (Fig. 4). The presence of MgATP did not change the unfolding behavior of Hsp90, indicating that Hsp90 is not stabilized by ATP.

Addition of ATP Does Not Influence the Intrinsic Fluorescence of Hsp90—Previously it had been reported that addition of ATP decreases the intrinsic fluorescence of Hsp90 (Csermely *et al.*, 1993). We readdressed this question again by comparing Hsp90 with the ATP-binding protein Hsc70 and the non-ATP-binding proteins BSA and Fab fragment concerning changes in the intrinsic fluorescence in the presence of ATP.

We found that addition of increasing concentrations of ATP results in a decrease of the intrinsic protein fluorescence independent of the protein used. This is due to the "inner filter effect" of ATP, *i.e.* the light absorption of ATP leading to a decreased fluorescence emission. It is possible to correct fluorescence spectra recorded in the presence of ATP for this non-specific effect of the nucleotide, as described by Birdsall *et al.* (1983). This allows to detect potential specific influences of ATP on the intrinsic fluorescence of proteins. After correction, as shown in Fig. 5, a slight decrease in fluorescence accounting for about 5% of the signal was observed with all proteins studied. Most importantly, however, both Hsp90 and the non-ATP-binding protein Fab gave a constant fluorescence signal at ATP concentrations, ranging from 2 μM to 2.5 mM, while the intrinsic fluorescence of Hsc70 increased by more than 10%, reflecting the specific binding of ATP. In the case of BSA, the amplitude decreased by about 25% upon increasing ATP concentrations, most likely due to unspecific interaction with ATP.

Fluorescence-labeled ADP Does Not Bind to Hsp90—Binding of ATP to proteins does not necessarily lead to changes in the intrinsic fluorescence of a protein. Therefore, we performed experiments in which we used fluorescent ADP analogues to monitor binding. Here, specific binding results in a fluorescence change of the label. Increasing amounts of Hsp90 were incubated with a fixed amount of the ligand and the fluorescence emission was recorded. The experiments showed that the fluorescence of a 0.4 μM solution of the nucleotide analogues MABA-ADP and MANT-ADP increases only slightly (by some 3% and 0%, respectively) when up to 0.85 μM Hsp90 was added (Fig. 6). In contrast, control experiments with DnaK (Hsc70 from *E. coli*) show the fluorescence of MABA-ADP to increase by some 250% when up to 0.8 μM of the ATP-dependent molecular chaperone was added (Fig. 6). This is indicative of a high affinity of DnaK for MABA-ATP. Furthermore, in this case cold ADP could compete for binding of the labeled analogue resulting in a constant fluorescence signal despite increasing MABA-ADP concentrations.The 3% increase in fluorescence emission of MABA-ADP observed in the presence of Hsp90 could not be competed by cold ADP. Similar experiments using the ADP analogue etheno-ADP showed only a minor fluorescence increase upon addition of Hsp90. Again, the small fluorescence change in the presence of Hsp90 could not be competed by cold ADP (data not shown).

Low ATPase Activity of Hsp90 Preparations Is Similar to Background Values Obtained for Other Proteins—In addition to ATP binding, previous reports also suggested that isolated Hsp90 may be an ATP hydrolyzing enzyme (Nadeau *et al.*, 1991, 1992). We, however, had failed to detect any ATPase activity in highly purified bovine Hsp90 preparations (Wiech *et al.*, 1993). Here, we have reinvestigated the ATPase activity of the yeast Hsp90 preparation, which we had been using for the ATP binding experiments. Using radiolabeled ATP we detected a low ATP hydrolyzing activity (Table I). This activity was severalfold lower than that of BiP, an endoplasmic member of the Hsc70 family, which possesses a very weak ATPase (Table I). We calculated that a potent phosphatase or kinase, such as casein kinase II present in levels as low as 0.01% in protein preparations could be responsible for this effect. To test if ATPase activity can be detected in highly purified commercially available proteins, we assayed for ATPase activity in "ultrapure RNase" (Table I). This preparation was found to contain similar levels of ATPase as our highly purified Hsp90. Furthermore, a purified DnaK mutant, which was shown to be catalytically inactive, gave values under steady state conditions that were similar to those obtained here for Hsp90.[3] These results confirm earlier suggestions that the ATPase activity of Hsp90 from various *Trypanosoma* species could be due to minor impurities of the protein preparation (Shi *et al.*, 1994).

Sequence Analysis of Hsp90 Does Not Reveal ATP Binding Motifs—Previous analysis of the sequence of murine Hsp90 showed the presence of a shortened version of the conserved

[3] J. Reinstein, personal communication.

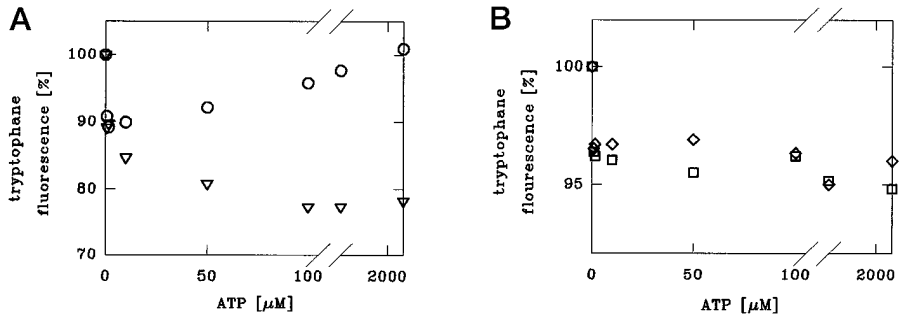

FIG. 5. **Influence of ATP on the intrinsic fluorescence of Hsp90, Hsc70, BSA, and IgG.** ATP-induced fluorescence changes of (*A*) 70 μg/ml bovine Hsc70 (○) and 57 μg/ml BSA (▽), (*B*) 128 μg/ml yeast Hsp90 (□) and 128 μg/ml IgG (◇) in the presence of various concentrations of ATP were monitored at 335 nm (Hsc70), 330 nm (BSA), and 325 nm (Hsp90 and IgG) after incubation for 1 min. The inner filter effect of ATP was calculated as described under "Materials and Methods."

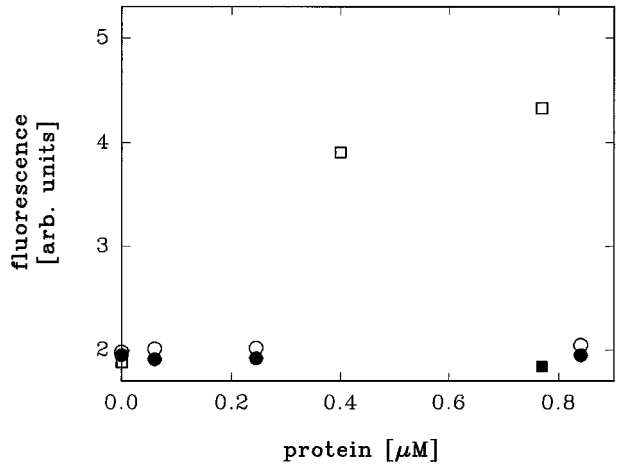

FIG. 6. **Binding of labeled ADP analogues to Hsp90 and DnaK.** Titration of yeast Hsp90 against 0.4 μM MABA-ADP (○) and 0.4 μM MANT-ADP (●) as well as DnaK titration against 0.4 μM MABA-ADP (□). The fluorescence change of the labeled analogue upon addition of the respective protein was monitored. ■, fluorescence change after addition of 1 mM ADP in the presence of 0.75 μM DnaK and 0.4 μM MABA-ADP.

Walker type ATP binding motifs A and B (Csermely and Kahn, 1991). We readdressed this question by searching the complete SwissProt data base for type A and B consensus sequences of ATP binding motifs (Walker *et al.*, 1982; Chin *et al.*, 1988). The search pattern used identified 622 sequences containing the complete type A motif. Most of these proteins are known ATPases or kinases. Hsp90 was not found using this search pattern. Chaperones identified in this search revealed a consensus sequence, which is slightly modified compared to that of kinases or ATP synthases (Table II, part A). Hsp90, unlike ATPases and kinases but like IgG or lysozyme, contained only the G-K-(T/S) motif, which is only part of the complete type A motif. In total, 8937 sequences (20.6% of *all* proteins in SwissProt data base) revealed this tripeptide sequence, reflecting the high statistical probability for the occurrence of this tripeptide sequence.

A selected set of proteins possessing either the correct type A consensus sequence or just the G-K-(T/S) tripeptide was screened for the type B consensus sequence. This motif is quite unspecific and thus is only a good indication for the presence of an ATP binding site if found together with a conserved type A sequence (Chin *et al.*, 1988). Table II (part B) compares the presence of type A and type B binding motifs in proteins with ATP binding properties. While proteins containing both motifs are unambiguously binding ATP, those lacking type A sequences exhibit no known ATP binding activity. Hsp90 belongs to this group of proteins, thus making it rather unlikely that Hsp90 contains an ATP binding site of the Walker type.

TABLE I
ATPase activity of yeast Hsp90 and reference proteins

The ATPase activity was determined as described. The specific activity is expressed as picomoles of ATP hydrolyzed/minute and mg of protein at 37 °C.

Proteins	Specific activity
	$pmol \cdot min^{-1} \cdot mg^{-1}$
Casein kinase II[a]	5040
BiP[b]	2.2
RNase A	0.4
Bovine Hsp90[c]	<0.1
Yeast Hsp90 (1 mM $MgCl_2$)	0.6
Yeast Hsp90 (1 mM $MnCl_2$)	0.7
Yeast Hsp90 (1 mM $CaCl_2$)	0.2
Yeast Hsp90 (10 min, 95 °C)	<0.1

[a] Specific activity of highly purified caseine kinase II at 30 °C (Litchfield *et al.*, 1990).
[b] G. Knarr, University of Regensburg, personal communication; Knarr *et al.* (1995).
[c] Wiech *et al.* (1993).

DISCUSSION

In recent years increasing interest has developed concerning the structure and function of the highly abundant cytosolic protein Hsp90. It is known that Hsp90 is involved in modulating the structure of target proteins, acts in concert with several heat shock and non-heat shock proteins *in vivo* and functions as a general molecular chaperone *in vitro* (*cf.* Jakob and Buchner, 1994). However, no consensus has yet been reached concerning the question whether or not ATP binding plays a role in the functional mechanism of Hsp90. While the *in vitro* chaperoning function of isolated Hsp90 is clearly ATP-independent (Wiech *et al.*, 1992; Shaknovich *et al.*, 1992; Shue and Kohtz, 1994; Schumacher *et al.*, 1994; Jakob *et al.*, 1995a; Miyata and Yahara, 1995), ATP binding and ATPase activity of Hsp90 have been reported recently (Csermely & Kahn, 1991; Csermely *et al.*, 1993; Nadeau *et al.*, 1992, 1993). Therefore, it is of crucial importance to study the potential influence of ATP on the structure and function of Hsp90 in more detail. We have addressed this question by studying the binding of MgATP to purified Hsp90. The first step required was to analyze whether any nucleotides were bound to purified Hsp90. Using two independent methods, isolated Hsp90 was shown to be nucleotide-free. This was a necessary prerequisite for subsequent binding studies, since the failure to detect ATP binding could therefore not be attributed to preoccupied nucleotide binding sites. Next, we reexamined experiments, the results of which had previously been interpreted as evidence for specific binding of MgATP to Hsp90. Given that Hsp90 interacts with nonnative substrate proteins as well as with native partner proteins, isolated Hsp90 can be considered to expose several reactive binding sites. Therefore, it was important to differentiate between specific binding of ATP to nucleotide binding sites and unspecific interactions of ATP with the protein surface. Based on these considerations, we compared the effects of ATP on Hsp90 with the effects of ATP on known ATP-binding and

TABLE II

Comparison of ATP-binding sequences

Type A and type B sequences denote the putative triphosphate- and adenine-binding sequences, respectively (Walker *et al.*, 1982; Chin *et al.*, 1988). O represents hydrophobic residues. The data base SwissProt was used for alignment of protein sequences using FINDPATTERNS. The total number of examined sequences was 43,470. A total of 8937 sequences contain the GKT/S motif and 622 sequences a type A-consensus sequence. The selected sequences were then analyzed for the presence of a type B-consensus sequences using FINDPATTERNS.

A Organism	Protein	Start	Sequence
		amino acid	
	Type A-consensus		**A/G**-*X*-*X*-*X*-*X*- **G-K-T/S**-*X*-*X*-*X*-*X*-*X*-*X*-**V/I**
H. sapiens	Serine/threonine-protein kinase	182	A -*X*-*X*-*X*-*X*- **G-K-S** -*X*-*X*-*X*-*X*-*X*- *V*
B. taurus	ATP-synthase α chain	217	G -*X*-*X*-*X*-*X*- **G-K-T** -*X*-*X*-*X*-*X*-*X*- I
S. cerevisiae	Hsp104	217	G -*X*-*X*-*X*-*X*- **G-K-T** -*X*-*X*-*X*-*X*-*X*-V- *X*
A. thaliana	Cpn60	208	G-*X*-*X* -*X*-*X*-*X*-*X*- **G-K-T** -*X*-*X*-*X*-*X*-*X*- V
B. taurus	PPI	30	G-*X*-*X*-*X* -*X*-*X*-*X*-*X*- **G-K-T** -*X*-*X*-*X*-*X*-V-*X*- *X*
E. electricus	Hemoglobin α chain	60	A -*X*-*X*-*X*-*X*- **G-K-T** -*X*-*X*-*X*-*X*-*X*- *X*-*X*-V
M. musculus	Ig heavy chain	82	*X* -*X*-*X*-*X*-*X*- **G-K-S** -*X*-*X*-*X*-V-*X*-*X*- *X*
H. sapiens	Hsp90 α	537	*X* -*X*-*X*-*X*-*X*- **G-K-T** -*X*-*X*-*X*-V-*X*-*X*- *X*
S. scrofa	lysozyme c-1	68	*X* -*X*-*X*-*X*-*X*- **G-K-T** -*X*-*X*-*X*-V-*X*-*X*- *X*
B. taurus	Hsc70	52	*X* -*X*-G-*X*-*X*- G-K-T *X*-*X*-*X*-*X*-V X
	Type B-consensus		**H/R/K**- X_{5-8} -**O**-*X*-**O**-**O**-**D/E**
H. sapiens	Serine/threonine-protein kinase	231	**H**-*X*-*X*-*X*-*X*-*X*-*X*-*X*-*X*-**F**-E-**Y**-**L**-**D**
B. taurus	ATP-synthase α chain	31	**K**-*X*-*X*-*X*-*X*-*X*-*X*-*X*- **I**-Y-G-**I**-**E**
S. cerevisiae	Hsp104	392	**R**-*X*-*X*-*X*-*X*-*X*- **L**-D-**L**-**V**-**D**
A. thaliana	Cpn60	261	**K**-*X*-*X*-*X*-*X*-*X*-*X*-*X*- **V**-K-**V**-**L**-**E**
B. taurus	PPI	13	**K**-*X*-*X*-*X*-*X*-*X*-*X*-*X*-*X*-**L**-R-**L**-G-**D**
E. electricus	Hemoglobin α chain	63	**K**-*X*-*X*-*X*-*X*-*X*-*X*- G-G-**I**-**A**-**E**
M. musculus	Ig heavy chain	23	**H**-*X*-*X*-*X*-*X*-*X*-*X*- **Q**-S-G-**A**-**E**
H. sapiens	Hsp90 α	366	**K**-*X*-*X*-*X*-*X*-*X*- **V**-F-**I**-**M**-**D**
S. scrofa	lysozyme c-1	81	**H**-*X*-*X*-*X*-*X*-*X*- **L**-L-D-D-**D**
B. taurus	Hsc70	188	K-*X*-*X*-*X*-*X*-*X*-*X*- V-L-I-F-D

B Organism	Protein	Consensus		ATP binding
		Type A	Type B	
H. sapiens	Serine/threonine-protein kinase	+	+	+
B. taurus	ATP-synthase α chain	+	+	+
S. cerevisiae	Hsp104	+/−	+	+
A. thaliana	Cpn60	+/−	+	+
B. taurus	PPI	+/−	+	?
E. electricus	hemoglobin α chain	+/−	+	−
M. musculus	Ig heavy chain	−	+	−
H. sapiens	Hsp90 α	−	+	?
S. scrofa	lysozyme c-1	−	−	−
B. taurus	Hsc70	+/−	+	+

non-ATP-binding proteins. We performed cross-linking experiments of the respective proteins with azido-ATP. Specific association of MgATP could only be detected for the ATP-dependent chaperone Hsc70. The observation that cross-linking of proteins with azido-ATP can also occur in a nonspecific way is in good agreement with a previous report by Todd and co-workers (1995). Nonspecific cross-linking is based on the formation of long lived reactive azido-ATP species. In addition, we performed titration studies with labeled ADP analogues, in which the specific binding leads to a fluorescence change of the label, independent of the distribution of aromatic amino acids in the protein. With the ADP analogues used, no significant fluorescence changes could be observed upon addition of Hsp90. In contrast, DnaK, the prokaryotic Hsc70 homologue, showed large changes in fluorescence. This suggested to us that Hsp90 is not an ATP-binding protein.

Confirming these results, we were unable to detect binding of Hsp90 to immobilized ATP and we did not see any stabilizing effect of ATP on the unfolding transition of Hsp90. Finally, we performed ATPase measurements with Hsp90. In contrast to bovine Hsp90 (Wiech *et al.*, 1993), low ATPase activities could be detected in some yeast Hsp90 preparations. However, ATPase assays are very sensitive to the presence of traces of highly active, contaminating ATPases, phosphatases, and kinases, which can result in the detection of low ATPase activities in many purified protein preparations. One example of this is an inactive DnaK variant, mutated so to eliminate its ATPase active site, which, even when purified according to different protocols, gave low ATPase activities similar to the ones obtained for Hsp90.[3] Rates of <0.7 pmol·min^{-1} mg^{-1} apparently represent typical background values for ATPase activities in protein preparations. Thus extreme care is needed before low ATPase activities can be attributed to any particular purified protein.

Based on these results and considering the analysis of the amino acid sequence, which did not reveal significant similarity with known ATP-binding motifs, we can certainly exclude the proposed tight binding of ATP to Hsp90 (*cf.* Table III) as well as the high levels of ATPase activity of Hsp90, previously reported (Nadeau *et al.*, 1992, 1993). Whether nucleotides can bind with very low affinity to Hsp90 cannot be completely ruled out based on negative results but in the light of the consistent data obtained with different assay systems this seems rather unlikely. In addition, we cannot completely exclude the possibility that binding of additional cofactors (partner proteins, peptides, transition state metals) may modulate the structure of Hsp90 to induce ATP binding. Addressing this question will require a more detailed understanding of the interaction of Hsp90 with these potential modulators.

In this context, the general question whether ATP hydrolysis is a necessary prerequisite for efficiently chaperoning protein folding arises. The increasing number of chaperones, whose

TABLE III
Hsp90 is not an ATP-binding protein

Method	Hsp90	BSA/IgG	Hsc70/DnaK
Binding to ATP agarose	−	−	+
Cross-linking with azido-ATP	+	+	+
Competition with cold ATP	> 5 mM	> 5 mM	≥ 50 μM
Influence of ATP on the stability toward chemical denaturation	−	ND	+[a]
Influence of ATP on the intrinsic fluorescence	−	−	+
Binding of fluorescence-labeled ADP	−	ND	+
Presence of ATP binding motifs in amino acid sequence	−	−	+

[a] Palleros *et al.* (1993).

function does not require ATP such as small Hsps, DnaJ, SecB, PapD, and calnexin/calreticulin argues strongly against this notion. The functional mechanism seems to vary between different members of these ATP-independent chaperones; however, all of them recognize nonnative polypeptides and are able to bind and release them in the absence of ATP. Although ATP and the co-chaperone GroES are required for the efficient release of nonnative proteins from the ATP-dependent chaperone GroEL under non-permissive folding conditions, it was recently demonstrated that the underlying cycles of binding and release are independent of ATP (Schmidt *et al.*, 1994; Sparrer *et al.*, 1996). Specifically, ATP decreases mainly the microscopic on-rate of GroEL for nonnative protein, which results in an efficient release of GroE-bound protein because rebinding is prevented (Sparrer *et al.*, 1996).

In the case of Hsp90, binding cycles for nonnative protein similar to the observed ATP-independent binding cycles of GroEL have been postulated (Jakob *et al.*, 1995a). It was further suggested that Hsp90 interacts preferentially with structured nonnative proteins. The conversion to the native form can be induced either by ligand binding (in the case of receptors), by myristylation (in the case of kinases) or by a folding reaction (in the case of folding and unfolding intermediates). Since external factors seem to drive the final conversion of the substrate protein, modulation of the binding cycles of Hsp90 by ATP is not required. Simply binding, release, and rebinding are sufficient to exert the Hsp90 function. In agreement with this hypothesis, all *in vitro* studies with purified Hsp90 showed that ATP is not required for the functional interaction of Hsp90 with nonnative proteins such as citrate synthase, MyoD, casein kinase II, and luciferase (Wiech *et al.*, 1992; Jakob *et al.*, 1995a, 1995b; Shaknovich *et al.*, 1992; Miyata and Yahara, 1995; Schumacher *et al.*, 1994). However, considering the action of Hsp90 as a member of the so-called "super chaperone complex," what is then the role of Hsc70 and the partner proteins? Little is known about their function, but it was suggested that Hsc70 together with p60 mediates the formation of the Hsp90·Hsp56·p23·steroid receptor complex. The mechanism of this ATP-driven complex formation is not yet understood, but Hsc70 seems to be the likely candidate responsible for the observed ATP requirement of this process. Modulators and cofactors like molybdate may also be involved (Johnson and Toft, 1995). Additionally, the idea has been proposed that Hsp90 and the Hsc70/DnaJ system share functions in the folding of proteins. In this scenario, the role of the ATP-independent Hsp90 would be to "hold" the nonnative protein, while Hsc70 in cooperation with DnaJ would "fold" the protein in an ATP-dependent way (Bohen *et al.*, 1995).

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