Conserved Conformational Changes in the ATPase Cycle of Human Hsp90*

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The dimeric molecular chaperone Hsp90 is required for the activation and stabilization of hundreds of substrate proteins, many of which participate in signal transduction pathways. The activation process depends on the hydrolysis of ATP by Hsp90. Hsp90 consists of a C-terminal dimerization domain, a middle domain, which may interact with substrate protein, and an N-terminal ATP-binding domain. A complex cycle of conformational changes has been proposed for the ATPase cycle of yeast Hsp90, where a critical step during the reaction requires the transient N-terminal dimerization of the two protomers. The ATPase cycle of human Hsp90 is less well understood, and significant differences have been proposed regarding key mechanistic aspects. ATP hydrolysis by human Hsp90α and Hsp90β is 10-fold slower than that of yeast Hsp90. Despite these differences, experiments suggest that the underlying enzymatic mechanisms are highly similar. In both cases, a concerted conformational rearrangement involving the N-terminal domains of both subunits is controlling the rate of ATP turnover, and N-terminal cross-talk determines the rate-limiting steps. Furthermore, similar to yeast Hsp90, the slow ATP hydrolysis by human Hsp90s can be stimulated up to 100-fold by the addition of the co-chaperone Aha1 from either human or yeast origin. Together, our results show that the basic principles of the Hsp90 ATPase reaction are conserved between yeast and humans, including the dimerization of the N-terminal domains and its regulation by the repositioning of the ATP lid from its original position to a catalytically competent one.

Furthermore, it has been shown that ATP hydrolysis is required for the essential function of Hsp90 in yeast (6, 7). Hsp90 is a dimeric protein of identical subunits (8). Recent crystal structures of Hsp90 proteins from Escherichia coli (9, 10), yeast (11), and the endoplasmic reticulum (12) from dogs have shown that the basic domain organization is conserved. The N-terminal domain (N-domain) binds ATP. This domain is followed by a linker region of variable length in different organisms, which connects the N-domain with the middle domain (M-domain). The C-terminal domain (C-domain) associates with its partner domain in the other subunit to form the dimer. Nevertheless, the positioning of the N-, M-, and C-domains relative to each other is dramatically different in the crystal structures.

An ATPase reaction cycle has been proposed based on structural and kinetic studies on yeast Hsp90 (13, 14). ATP-binding by Hsp90 triggers a ring-shaped conformation, in which the N-domains interact with each other via a strand exchange mechanism, in addition to the permanent dimerization of the C-domains. This is achieved by exchanging the first β-strand and the first α-helix between the two N-domains. This conformation, which is required for productive ATP hydrolysis, had been proposed for yeast Hsp90 on the basis of biochemical experiments (13, 15), and recently it was also directly shown by x-ray crystallography (11). Both crystallographic and biochemical data show in addition that a lid structure (“ATP lid”) within the N-domain closes over the nucleotide binding site upon ATP binding, and this movement initiates the N-terminal dimerization reaction (11, 16). For other Hsp90 homologues, this conformation has not been demonstrated unambiguously. Furthermore, for human Hsp90, evidence exists that it does not even form this conformation, and instead both subunits hydrolyze ATP in an uncoordinated manner (17). Besides the proposed differences in the ATP hydrolysis cycle, differences have also been proposed for the interaction of yeast and mammalian Hsp90 with their co-chaperones (18). Co-chaperones are an important part of the eukaryotic Hsp90 machinery, required for the maturation of client proteins. Some co-chaperones have been identified as regulators of ATP turnover. In yeast, the co-chaperones Sba1 and Sti1 both inhibit the ATPase activity (19,

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The molecular chaperone Hsp90 is a critical activator of many signaling proteins (1–4). Among its substrates are protein kinases, transcription factors, and a wide variety of unrelated proteins, which have been identified during the last decades. At least in the well studied cycle of steroid hormone receptor activation, it has been shown that the binding and hydrolysis of ATP by Hsp90 are required to convert the glucocorticoid receptor to its active, hormone binding form (5).
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20), and Aha1 accelerates turnover (20). Sti1 and Sba1 bind to specific conformations of Hsp90 during the ATP hydrolysis cycle. Sti1 binds to Hsp90 in the open conformation with the N-domains not being associated (21), whereas Sba1/p23 binds to the closed conformation in which the N-domains are dimerized (13, 22, 23). In human Hsp90, these co-chaperones appear to act differently. Hop, the Sti1 homologue, apparently does not influence the ATPase activity of human Hsp90 (18, 24), and p23, the Sba1 homologue, is thought to bind independently to both protomers in a conformation that does not necessarily require N-terminal dimerization (17, 18).

This leaves us with the puzzling situation that the ATPase cycle of human Hsp90 seems to be different from that of other family members, such as yeast Hsp90 and the recently analyzed Grp94 (12, 25). Here, we set out to reinvestigate the ATPase mechanism of human Hsp90 with a view to determine to what extent similarities exist between the mechanisms of ATP turnover in yeast Hsp90 and in human Hsp90. Previously, we had established several specific assays for the analysis of the Hsp90 ATPase, including the formation and analysis of Hsp90 heterodimers (14, 16). This allowed us to combine different Hsp90 constructs in one dimer and analyze the contribution of specific elements or domains to its ATPase activity. We employed this approach here to determine whether structural elements in human Hsp90 function in a similar manner, when compared with yeast Hsp90. According to our data, the basic principles, including the N-terminal dimerization and the mechanism of the ATP lid, are conserved between the two eukaryotic proteins. In addition, also the mechanism of interaction with the co-chaperone Aha1 and its stimulatory efficiency is conserved between species. Our results finally also allow us to draw conclusions regarding the basis for the dramatically slower turnover rate of human Hsp90, compared with its yeast homologue.

MATERIALS AND METHODS

Protein Constructs—Constructs for bacterial expression were based on the plasmids pET28b-yAha1, pET28b-hAha1, pET23-Hsp90α, and pET9-Hsp90β. Hsp90β was also purified from baculovirus-infected insect cells as described previously (26). Yeast Aha1 was cloned after PCR amplification from yeast DNA into the corresponding expression vector by recombinant DNA technology. Plasmids for human Aha1 and both human Hsp90 homologues were a kind gift from David Toft (Mayo Clinic, Rochester, MN). All of the plasmids were sequenced, and the sequence was confirmed to correspond to the sequence in the public data bases. Human Hsp90α matched the genome sequence BC108695, and human Hsp90β matched the genome sequence P08238.

The deletion constructs His6-lidless human Hsp90α (hHsp90α), His6-lidless hHsp90β, His6-MC-hHsp90α, His6-C-hHsp90β, and His6-MC-hHsp90β were generated by PCR using the full-length plasmid sequences. For lidless hHsp90α and lidless hHsp90β, the amino acid stretches covering the ATP lid (Ser113–Gly137 and Thr110–Gly132, respectively) were replaced by a Ser-Gly-Ser linker. Lidless constructs were generated by linker-PCR and cloned into the pET28b expression plasmid using NheI and NotI restriction sites. MC-constructs (starting at amino acid Lys278 and amino acid Lys270 for hHsp90α and hHsp90β, respectively) and the C-construct for hHsp90β (from amino acid 537 to 724) were generated by PCR amplification and inserted into the pET28b expression vector by NdeI/NotI (MC-hHsp90β and His6-C-hHsp90β) and NheI/NotI (MC-hHsp90α).

Protein Purification—Proteins were purified from heterologous expression cultures. Hsp90β was purified from baculovirus-infected insect cells as described previously (27). In addition, Hsp90β (and also Hsp90α) were expressed from plasmids (pET23-Hsp90α and pET9-Hsp90β) in the E. coli strain BL21 (DE3) RIL (Stratagene, La Jolla, CA). Cells were grown at 37 °C in LB medium. Hsp90 expression was induced at an A600 of 0.7. Expression was allowed to proceed for 4 h. Cells were harvested by centrifugation, resuspended in 40 mM HEPES/KOH, pH 7.5, 20 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM EDTA buffer, and lysed using a cell disrupter (Constant Systems, Weehawken, UK). The cleared lysate was applied to a DEAD-Sepharose column (GE Bioscience). Hsp90-containing fractions were loaded on to a ResourceQ column (GE Bioscience). As a final purification step, a gel filtration column (Superdex 200HiLoad; GE Bioscience) was performed in 40 mM HEPES/KOH, pH 7.5, 300 mM KCl, 1 mM EDTA, 1 mM DTT.

All other Hsp90 constructs and both Aha1 proteins were expressed in E. coli as described above and purified using an N-terminal His6 tag for affinity purification on a nickel-loaded chelating Sepharose matrix (GE Bioscience). Protein-containing fractions were further purified by ion exchange chromatography using a ResourceQ column (GE Bioscience) and a gel filtration step as described above.

The purified proteins were subjected to mass spectrometry to confirm the size of the proteins. Protein purity was higher than 95% in all cases. Protein concentrations were determined using the calculated extinction coefficients of ε280 = 0.70 for hHsp90α, ε280 = 0.70 for hHsp90β, ε280 = 1.71 for hAha1, and ε280 = 1.28 for yAha1. Hsp90 proteins were stored in 40 mM HEPES, pH 7.5, 20 mM KCl, 1 mM DTT at concentrations of 3–5 mg/ml, and Aha1 was stored in the same buffer at 10–20 mg/ml at −80 °C.

Analytical Size Exclusion Chromatography—Analytical size exclusion chromatography was performed on a Jasco PU1580 HPLC system equipped with a Jasco FP 1520 fluorescence detector (Jasco, Gross-Umstadt, Germany) using a Superdex 200HR (GE Bioscience) column in 40 mM HEPES/KOH, 150 mM KCl, 1 mM DTT as running buffer. Protein concentrations ranging from 50 µM to 10 µM were applied, and the elution time was monitored by intrinsic protein fluorescence at an excitation wavelength of 280 nm and an emission wavelength of 320 nm.

ATPase Measurements—ATP turnover rates were determined by a spectrophotometric assay with an enzymatic ATP regeneration system in 40 mM HEPES/KOH, pH 7.5, 150 mM KCl, 1 mM DTT, 5 mM MgCl2, and 2 mM ATP as described elsewhere (6, 14, 28). Hsp90 concentrations of 1–10 µM were used, and the temperature was set to 37 °C. The addition of 50 µM radicicol was used to specifically inhibit the Hsp90 ATPase activity in order to accurately determine the ATP turnover of Hsp90. For ATPase assays with Aha1, KCl was omitted to improve the affinity between Hsp90 and Aha1, which is very salt-sensitive (20). Heterodimer formation during the ATPase
Determination of the $K_m$ value for ATP was performed by varying the ATP concentration while keeping other parameters of the ATPase measurement (buffer conditions, temperature, and Hsp90 concentration) constant. The $K_m$ value then was derived from the ATPase activities according to Equation 1, with $c$ being the concentration of ATP in the assay.

$$V = V_{\text{max}} \times c/(c + K_m)$$  \hspace{1cm} \text{(Eq. 1)}

Determination of the activation energies was performed by varying the temperature of the ATPase assay. ATPase activities were plotted as $\ln(v)$ against $1/T$ and analyzed regarding the linear nature of the plot. At temperatures above 55 °C, all three proteins showed loss of activity. The linear part of the plot was analyzed based on Equation 2, resulting in a value for the activation energy $E_A$.

$$\ln(v) = \text{Const} - E_A/R \times (1/T)$$  \hspace{1cm} \text{(Eq. 2)}

Subunit Exchange Kinetics—Subunit exchange in the Hsp90 dimer was monitored by changes in ATPase activity upon the addition of either activating or inactivating Hsp90-subunits. To this end, 20 μL of a solution containing either MC-hHsp90β or lidless hHsp90β were added to an ATPase assay mixture of 120 μL. The change in ATPase activity was monitored at 340 nm at the indicated buffer conditions and temperature. Data were processed by multiplying the absorption traces after the addition of the respective Hsp90 variant by a factor of 1.167 (140/120) to correct the reduced absorption itself and the activity, which was reduced due to the change in concentration of active Hsp90. Data were analyzed according to Equation 3,

$$A_{340} = A_0 + (m_{\text{final}} + (m_{\text{start}} - m_{\text{final}}) \times e^{-k \times t}) \times t \hspace{1cm} \text{(Eq. 3)}$$

where $m_{\text{start}}$ and $m_{\text{final}}$ represent the slope of the ATPase assay prior to and after subunit exchange, respectively, and $k$ is an observed first-order rate constant, which includes the initial dissociation of the subunits and the reassociation to form the active species. Therefore, the interpretation of this value is not straightforward; nevertheless, it can be used to compare the overall dynamics of the system (14). As a control, we added 20 μL of buffer and observed a reduced absorbance and a slope corresponding to the dilution factor but no observable reaction leading to further changes in the turnover rate.

Single-turnover ATPase Assay—For single-turnover measurements, we used hHsp90β at concentrations of 321 μM. ATP was added to a concentration of 260 μM, with 1.5 mCi of [α-32P]-ATP (Hartmann Analytics, Braunschweig, Germany). The assay was performed in 40 mM Hepes, pH 7.5, 150 mM KC1, 5 mM MgCl2, at 37 °C and started by the addition of ATP. The hydrolysis kinetics were measured by taking samples at defined time points. The reaction was stopped by the addition of 50 mM EDTA, pH 8.0. Samples were subjected to thin layer chromatography, and the radioactivity was analyzed using a Typhoon PhosphorImager (GE Biosciences), as described (25, 29). Chase experiments were performed by adding a 100-fold excess of cold ADP over ATP after 5 min of hydrolysis time. Proceeding hydrolysis was analyzed again by taking samples at defined time points, stopping the reaction with 50 mM EDTA and analyzing the amount of hydrolyzed ATP by thin layer chromatography. Any hydrolysis of radioactive ATP after the addition of ADP is considered to be the result of commitment during the ATPase cycle.

Kinetic Evaluation of Nucleotide Binding—(Pγ)-MABA-ATP was injected into a solution of hHsp90α or hHsp90β using a SF-61 DX2 double mixing stopped flow instrumentation (Hi-Tech Scientific, Salisbury, UK). The excitation slit was set to 1 nm, if not indicated otherwise. Nucleotide binding reactions were followed by direct excitation of the fluorescent MABA group at 364 nm. The emission was detected using a cut-off filter of 420 nm. Each experiment was performed between three and eight times, and the resulting time traces were averaged with the software from Hi-Tech Scientific for an accurate determination.

Different concentrations of Hsp90s were used to obtain multiple scans for the evaluation of both binding parameters, $k_{\text{on}}$ and $k_{\text{off}}$. In the case of hHsp90α, the determination of $k_{\text{off}}$ was performed independently by preincubating hHsp90 with (Pγ)-MABA-ATP. The fast addition of a large excess of ADP results in the direct observation of $k_{\text{off}}$. This value can be used to restrain the fit for $k_{\text{on}}$ and improve the accuracy of the determined values. $k_{\text{on}}$ is determined from the slope of the linear line in the plot ($\ln k_{\text{obs}}$ versus $c_{\text{Hsp90}}$). Concentrations are indicated as final concentrations in the instrument cell after dilution with ligand.

**RESULTS**

The ATPase Activity of Both Human Hsp90s Is Low and Salt-dependent—The ATPase activity of hHsp90β has been described to be very low (18). In order to determine the ATPase properties of both human Hsp90 homologues, recombinantly produced hHsp90α and hHsp90β were purified to homogeneity, and their basic enzymatic features were compared with yeast Hsp90 (yHsp90). We analyzed the ATPase activity at different salt concentrations and observed for both human proteins and yeast Hsp90 an increase in activity with increasing KCl concentrations (Fig. 1A). Apparently, for all three proteins, the rate-limiting step is sensitive to salt concentrations in agreement with the known salt dependence of GHKL-ATPases (30). However, for both human Hsp90 proteins, the turnover numbers were dramatically lower than those obtained for yeast Hsp90. Hydrolysis rate constants at standard salt concentrations (150 mM KCl) were 1.2 min⁻¹ for yHsp90 and 0.10 and 0.08 min⁻¹ for hHsp90α and hHsp90β, respectively. Both human Hsp90 homologues were sensitive to the Hsp90 inhibitors radicicol and geldanamycin (data not shown).

The $K_m$ values for yHsp90 and the two human Hsp90 proteins were determined to be in the range of 300 μM (Fig. 1B). This is in the range of previously determined $K_m$ values for other Hsp90 family members (31). Therefore, ATP affinity does not appear to be the cause for the slower ATP turnover rate of the human Hsp90s. Next, we analyzed the temperature dependence of the turnover rate in order to estimate the activation energy of the hydrolysis reaction. We determined the turnover
rates at 37, 45, 50, and 55 °C and plotted the results in a ln$(k)$ versus $1/T$ plot (Fig. 1C). This plot was used to estimate the activation energy of the hydrolysis reaction of hHsp90α, hHsp90β, and yHsp90. All Hsp90 members tested exhibit high activation energies of 130 kJ/mol for human Hsp90s and 91 kJ/mol for yeast Hsp90. The results imply that a significantly higher activation energy has to be overcome by human Hsp90s compared with yeast Hsp90, implying that their enzymatic efficiency is significantly lower. Nevertheless, for all three proteins, the unusually high activation energy may explain the slow turnover rates of 1.2 ATP/min (for yHsp90) or 0.10 and 0.08 ATP/min (for the human Hsp90α and β, respectively).

Conformational Changes Prior to Hydrolysis Represent the Rate-limiting Step of Human Hsp90—Given that human Hsp90s show decreased ATPase activities compared with the yeast protein, we wondered whether the nature of the rate-limiting step was conserved. To determine whether the rate of ATP binding was the rate-limiting step, we measured binding and dissociation kinetics using (Pγ)-MABA-ATP and determined a $k_{off}$ value of 4.1 s$^{-1}$ and a $k_{on}$ value of 0.12 μM$^{-1}$ s$^{-1}$ for human Hsp90β at 25 °C (Fig. 2A and Table 1). For hHsp90α, we observed similar values ($k_{off} = 3.13$ s$^{-1}$ and $k_{on} = 0.06$ μM$^{-1}$ s$^{-1}$). These values are similar to those previously determined for other Hsp90 family members (15, 25, 29).

Single-turnover ATPase measurements allow the direct determination of the $k_{cat}$ value, excluding influences of product release and the rebinding of ATP, which may contribute to the observed rate in steady state experiments. We performed single-turnover ATPase measurements with human Hsp90β in a similar manner as done before for yeast Hsp90 and Grp94 (25, 29) and observed a single-turnover rate constant of 0.07 min$^{-1}$ (Fig. 2B). This value is in good agreement with the rate constant observed under steady state conditions. This allows the conclusion that the rate-limiting step is either the hydrolysis reaction or events prior to hydrolysis. Since we knew already that nucleotide binding is fast, it is reasonable to assume that for hHsp90, either the hydrolysis reaction itself or conformational changes after ATP binding and prior to hydrolysis may be rate-limiting.
C-Hsp90 variant comprised solely of the dimerization domain hHsp90 minally dimerized conformation can also be observed for and crystallographically visualized (11). For hHsp90, this step was monitored via increasing MABA fluorescence after direct excitation of the fluorescent ATP analog. Data fol-

For hHsp90, it had been determined that these changes lead to a closed state in which a significant percentage of bound ATP is committed to hydrolysis after the initial binding event (29). When we performed these pulse-chase experiments with unlabeled ADP added after the single-turnover reaction was started. We were interested whether this structure contributes to the ATPase cycle of hHsp90 in a similar manner. To test this, we constructed a hHsp90β variant in which the putative lid was replaced by a short Ser-Gly-Ser stretch. When we tested this variant, we found that the deletion of the ATP lid leads to a complete loss of ATPase activity (data not shown). However, heterodimers between wild type Hsp90β and the lidless variant exhibited a strong increase in ATPase activity when compared with the wild type homodimer (Fig. 3B), suggesting that, as in yHsp90 (16), the ATP lid serves the function of a dimerization inhibitor for the N-domains and its removal results in an inactive but stimulatory protomer. These data provide strong evidence that interactions between the N-domains are important for the progression of the ATPase cycle of hHsp90β.

**N-terminal Interactions Are Required during Aha1-stimu-

**TABLE 1**

Kinetic values were determined by stopped-flow experiments using fluorescently labeled (Py)-MARA-ATP as described under “Materials and Methods.” The k_{off} values were obtained from analyzing a series of kinetic experiments with different Hsp90 concentrations. The k_{on} values were determined either by determination of the intercept (hHsp90β) or by chase experiments with excess ADP (hHsp90α). Experimental conditions were using a standard ATPase buffer at 25 °C.

|       | k_{on,25} | k_{off,25} | k_{D,25} |
|-------|-----------|------------|----------|
| hHsp90n | 31 ± 0.3  | 0.06 ± 0.04 | 51 |
| hHsp90β | 41 ± 0.3  | 0.12 ± 0.04 | 34 |
| yHsp90  | 2.0 ± 0.4^* | 0.13 ± 0.04^* | 15 |

^* Value obtained from Richter et al. (15).

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For yHsp90, it had been determined that these changes lead to a closed state in which a significant percentage of bound ATP is committed to hydrolysis after the initial binding event (29). These ATP molecules could not be exchanged with unlabeled ADP added after the single-turnover reaction was started. When we performed these pulse-chase experiments with hHsp90β, we found, in contrast to results with yHsp90, no detectable commitment during the hydrolysis reaction (Fig. 2B), implying that most of the ATP remains solvent-accessible during the rate-limiting step. The lack of ATP committed to hydrolysis after the initial binding reaction implies that the conformationally closed state either does not exist or is less efficiently populated in hHsp90 compared with yHsp90.

**N-terminal Dimerization and the Function of the ATP Lid Are Conserved in Human Hsp90**—For yHsp90, an N-terminal dimerized state had been biochemically demonstrated (13, 15) and crystallographically visualized (11). For hHsp90, this step has been challenged, and current models suggest a hydrolysis cycle devoid of this step (17). To directly test whether the N-termi-

**FIGURE 2. Determination of the rate-limiting step of Hsp90.** A, the kinetics of nucleotide binding to Hsp90β were analyzed at 25 °C in a buffer of 40 mM HEPES/KOH, pH 7.5, 150 mM KCl, 1 mM DTT, 5 mM MgCl₂ using (Py)-MABA-ATP as a fluorescent nucleotide analogue. Concentrations are indicated as final concentrations in the instrument cell after dilution with ligand. Various concentrations of hHsp90β were mixed with 1 μM (Py)-MABA-ATP in a stopped flow instrument. Hsp90β concentrations used were as follows: 10 μM (●), 8 μM (□), 5 μM (●), 4 μM (□), 2 μM (○), and 1 μM (●), and 0.5 μM (△). The figure depicts typical time traces. Binding was monitored via increasing MABA fluorescence after direct excitation of the fluorescent ATP analog. Data fol-

![Image](image-url)
is rather low (23% identity; supplemental Fig. 2). Both Aha1 homologues gave similar stimulation factors of 65-fold for human Hsp90α/H11022 and 80-fold for human Hsp90β/H11022. The observed similarities in the activation mechanism of yeast and human Aha1 proteins strongly imply a conservation of the hydrolysis mechanism, as the acceleration of ATP hydrolysis in most cases directly affects the rate-limiting step of the reaction. Therefore, we envision that identical conformational changes prior to the hydrolysis reaction are accelerated by the co-chaperone Aha1 in both hHsp90s and yHsp90.

We were further interested in whether the Aha1-stimulated ATPase activity can be influenced by the formation of heterodimers. Therefore, we added the N-terminal truncated hHsp90β mutant to hHsp90α in the presence of a large excess of Aha1. The addition of the truncated fragment resulted in a strong deceleration of the ATPase activity of hHsp90α. Already, equimolar concentrations of the truncated fragment were sufficient to significantly reduce the ATP turnover (Fig. 3C), implying that formation of defunct heterodimers results in the loss of ATPase activity also in the presence of the activator Aha1. These data suggest that not only the ATPase cycle in its unstimulated reaction requires the interaction between the N-domains of both protomers, but this reaction is also required during the Aha1-stimulated ATPase cycle.

**TABLE 2**

|                        | hHsp90α | hHsp90β |
|------------------------|---------|---------|
| **v**<sub>max</sub>    | 3.9 ± 0.3 | 4.3 ± 0.9 |
| **K**<sub>D</sub>      | 3.2 ± 0.4 | 6.5 ± 2.1 |
| **Factor**             | ~65     | ~86     |
| yAha1                  | 2.7 ± 0.4 | 5.8 ± 2.0 |
| hAha1                  | 2.6 ± 1.0 | 9 ± 3.5  |
| **Factor**             | ~45     | ~116    |

**FIGURE 3.** Formation of heterodimers modulates the ATP turnover of hHsp90. A, the addition of an excess amount of N-terminal deleted MC-Hsp90β (●) and C-Hsp90β (○) to Hsp90β results in a decreased ATP turnover rate due to the formation of heterodimers, which are characterized by a lower ATPase. ATPase assays were performed with 7 μM hHsp90β and varying amounts of MC-Hsp90β or C-Hsp90β in 40 mM HEPES/KOH, pH 7.5, 150 mM KCl, 5 mM MgCl₂, and 2 mM ATP at 37 °C. B, the addition of different concentrations of a mutant of Hsp90β, which has a Ser-Gly-Ser replacement instead of the ATP lid (lidless hHsp90β), stimulates the turnover of Hsp90β due to formation of heterodimers that hydrolyze ATP faster than the homodimeric Hsp90 variant. 3 μM hHsp90β was incubated with varying concentrations of lidless hHsp90β in 40 mM HEPES/KOH, pH 7.5, 20 mM KCl, 5 mM MgCl₂, and 2 mM ATP at 37 °C. C, 1 μM hHsp90β ATPase was stimulated with 20 μM yeast Aha in 40 mM HEPES/KOH, pH 7.5, 5 mM MgCl₂, 1 mM DTT. The addition of MC-hHsp90β, the ATPase activity was monitored.
To obtain further insight into dimerization, we used the potential of lidless hHsp90β to accelerate the ATPase activity of hHsp90β upon heterodimer formation. This allowed us to study subunit exchange kinetically by adding stimulatory lidless hHsp90β during ATPase assays. Upon the addition of lidless hHsp90β, we expected subunit exchange to occur and the ATPase activity to increase accordingly. Indeed, we observed an increase in the rate of ATP turnover, although the increase occurred with a rather slow kinetic, which hints at a rather slow subunit exchange in the Hsp90 dimer. The observed exchange rate of this reaction was fit to 0.022 min⁻¹ (Fig. 4A). The kinetics of subunit exchange thus proved to be slow compared with yHsp90, which was shown to fully exchange subunits within seconds in similar assays (14). Although the observed rate constant only provides a combined constant for the dissociation and reassociation of the dimers, it shows a less dynamic exchange of the C-domains of the human Hsp90 protein. As a control, we also added Aha1 to an ATPase reaction of hHsp90β and observed an immediate acceleration, in agreement with the notion that this effect is independent of subunit exchange (data not shown).

In a similar manner, we followed the consequences of N-terminally truncated hHsp90β added to wild type hHsp90β stimulated with Aha1. Again, we would expect to observe the formation of heterodimers and the coupled reduction in ATPase activity directly by changes in the slope of the ATPase assay. The kinetics we observed were similarly slow, with an observed rate constant of 0.085 min⁻¹ (Fig. 4B), implying that hHsp90β forms rather stable dimeric structures and that this characteristic is not changed by the presence of Aha1.

Dimerization Properties of hHsp90α and hHsp90β Are Different—Next we were interested whether heterodimer formation between α- and β-isomers of Hsp90 was possible, since both isoforms are present in the cell, at least under stress conditions (34).

We therefore tested whether hHsp90α variants can also be used to influence the ATPase activity of hHsp90β in heterodimer assays. Surprisingly, no changes were observed when lidless hHsp90α was added to hHsp90β (Fig. 5A). Additionally, we did not observe a reduction in ATP turnover of Aha1-stimulated hHsp90β after the addition of N-terminally truncated hHsp90α (Fig. 5B). Given that at least for hHsp90β the requirement for N-terminal interaction is already known, these data imply that the formation of heterodimers between hHsp90α constructs and hHsp90β did not occur at a level significant enough to inhibit this reaction.

We therefore tested whether subunit exchange in hHsp90α occurs in a similar way as in hHsp90β. We performed the heterodimer formation assays as described above using hHsp90α and lidless hHsp90α or lidless hHsp90β (Fig. 5A) and MC-hHsp90α or MC-hHsp90β (Fig. 5B). We could not observe influences of the lidless or the MC mutants (Fig. 5, A and B, respectively) on hHsp90α under conditions previously used for hHsp90β mutants in heterodimer assays with full-length Hsp90β. This implies that heterodimer formation between hHsp90α and hHsp90β or between hHsp90α and hHsp90α variants is strongly disfavored compared with hHsp90β. The reason might be that a very stable dimerization of hHsp90α precluded subunit exchange within the time frame of our analysis.

**FIGURE 4. Kinetics of subunit exchange of human Hsp90β.** A, subunit exchange was visualized by changes in the turnover rate of 2 μM hHsp90β upon the addition of 10 μM lidless hHsp90β. 40 mM HEPES/KOH, pH 7.5, 20 mM KCl, 5 mM MgCl₂, and 2 mM ATP was used as assay buffer. The arrow marks the time point of the addition of lidless hHsp90β (thick line) or buffer (thin line). The following curve has been corrected for the dilution, which occurred as a consequence of adding lidless hHsp90β or buffer. The kinetic parameters were derived from analyses as described under "Materials and Methods." B, subunit exchange was visualized upon the addition of 10 μM MC-hHsp90α to 1 μM preformed hHsp90α-yAha1 complexes; 40 mM HEPES/KOH, pH 7.5, 5 mM MgCl₂, and 2 mM ATP was used as assay buffer. The arrow marks the time point of the addition of MC-hHsp90α (thick line) or buffer (thin line). The following curve has been corrected for the dilution, which occurred as a consequence of adding MC-hHsp90α or buffer. The kinetic parameters were derived from analyses as described under "Materials and Methods."

**FIGURE 5. Heterodimer formation is diminished for hHsp90α.** A, lidless hHsp90α at different concentrations was added to 3 μM Hsp90β in order to form heterodimers. 40 mM HEPES/KOH, pH 7.5, 20 mM KCl, 5 mM MgCl₂, and 2 mM ATP was used as assay buffer. ATPase activities were normalized, and the change in activity upon the addition of either lidless hHsp90α or lidless hHsp90β to Hsp90α or Hsp90β was monitored. The heterodimer pairs used were hHsp90β/lidless hHsp90α (●), hHsp90β/lidless Hsp90β (○), hHsp90α/lidless hHsp90β (▲), and hHsp90α/lidless hHsp90α (▼). B, the ATPase of Hsp90β (1 μM) was stimulated in the presence of 20 μM yeast Aha1. 40 mM HEPES/KOH, pH 7.5, 5 mM MgCl₂, and 2 mM ATP was used as assay buffer. MC-hHsp90α or MC-hHsp90β (up to 2 μM) was added to the reaction, and ATP hydrolysis was monitored for both human Hsp90 homologues. The heterodimer pairs used were hHsp90β/MC-hHsp90β (●), hHsp90β/MC-hHsp90α (○), hHsp90α/MC-hHsp90β (▲), and hHsp90α/MC-hHsp90α (▼).
ATPase Cycle of Human Hsp90

In order to test whether the inability to form heterodimers with human Hsp90α fragments could be due to the reaction conditions used, we performed heterodimer analysis at an increased ionic strength and increased temperatures. We found that both an increase in ionic strength and in temperature resulted in a detectable subunit exchange, as evident by changes in ATP turnover rates (Table 3), implying that the lack of observable subunit exchange can be overcome by different conditions. These results allowed us also to determine the ATPase activity of Hsp90α-Hsp90β heterodimers. Our experiments show that these heterodimers have activities comparable with the respective homodimers tested under these conditions (data not shown).

The apparent differences at 37 °C in the dimerization behavior might explain earlier data by Minami et al. (8, 35), which had reported a lack of heterodimer formation between the otherwise nearly identical hHsp90α and hHsp90β and a lack of dissociation in hHsp90α (36).

DISCUSSION

ATP hydrolysis by the molecular chaperone Hsp90 is required for its in vivo function, as has been demonstrated in yeast (6, 7). This enzymatic reaction is coupled to large scale conformational changes. Whether this ATPase mechanism, which is supported both by biochemical and structural data (11, 16), can be generalized for the entire Hsp90 family is an important unresolved question. In this context, for the endoplasmic Hsp90 homologue Grp94, the prevailing notion was until recently that it binds but does not hydrolyze ATP (37). A detailed biochemical analysis showed, however, that Grp94 is an ATP-hydrolyzing enzyme with properties reminiscent of the well studied Hsp90 representative from the yeast cytosol (12, 25). Human Hsp90 is set apart from yeast Hsp90 and Grp94 in the sense that a low ATPase (18), and more importantly, crucial mechanistic differences between the two eukaryotic systems have been proposed (17). This challenges any attempt to develop a general model for the ATPase mechanism in the Hsp90 family. Furthermore, in the context of developing Hsp90 inhibitors for cancer therapy, a detailed description of the ATPase cycle of human Hsp90 seems required. The basic conundrum is whether comparable structural intermediates are formed during the ATPase cycle of both the yeast and the human protein. Although the yeast protein has been shown to dimerize N-terminally during the ATPase reaction, this state has been suggested not to be required for the ATPase cycle of human Hsp90 (16).

We show here that critical steps of the ATPase cycle are indeed conserved between eukaryotic Hsp90 species, independent of their turnover rates. We focused our attention on structural elements clearly demonstrated to be of importance during the reaction cycle of yeast Hsp90 (16). One well established assay is the analysis of heterodimeric Hsp90 species in which one wild type subunit in the dimer is replaced by a construct lacking the N-terminal nucleotide binding site. For hHsp90β, these heterodimers hydrolyze ATP with a reduced rate compared with symmetric wild type Hsp90 dimers. This result strongly suggests that N-terminal dimerization is also a crucial step in the reaction cycle of human Hsp90. Another regulatory element of interest is the so-called ATP lid, a structural element of the N-domain that changes its conformation after ATP binding. This change is required as a prerequisite for N-terminal dimerization, and additionally it plays an important role in catalysis. The replacement of the ATP lid with a Ser-Gly-Ser linker leads to a strongly stimulating but inactive Hsp90 protomer, both in yeast and human Hsp90. Taken together, the potential to form heterodimers with variants exhibiting different enzymatic properties indicates that the two protomers do not work independently of each other in the dimer; rather, they are closely involved in each other’s reaction mechanism. Therefore, our data show that the critical N-terminal interaction, which had been observed in yeast Hsp90, is conserved in the human Hsp90 system.

Having shown that the basic conformational changes associated with the ATP turnover are conserved, our data also allow zooming in on the rate-limiting step of the hHsp90-catalyzed ATPase cycle. Single-turnover experiments are required for detailed characterization of the rate-limiting step of an enzymatic reaction. The kinetic and single-turnover experiments performed in our study show that the rate-limiting step of the ATP hydrolysis reaction in human Hsp90 occurs after nucleotide binding but prior to the hydrolysis reaction. This in principle confirms conformational changes after the initial nucleotide binding reaction as the rate-limiting step. Since the nucleotide remains fully solvent-accessible during the rate-limiting step, this strongly argues that the N-terminal dimerization reaction, which would lead to a potential trapping of the nucleotide, is slow and therefore rate-limiting, as proposed for yeast Hsp90 (29), although, in contrast to yeast Hsp90, where about 30% of the ATP is trapped (29), we did not observe any enclosure or commitment of ATP for hydrolysis. Therefore, we see that the proportion of Hsp90 that is closed prior to hydrolysis is

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**TABLE 3**

Cross-stimulation of human Hsp90s with lidless constructs

Heterodimers between 2 μM hHsp90α and hHsp90β with 4 μM lidless constructs were formed under different conditions in order to identify factors that allow the formation of mixed α-β dimers. α-β dimers were formed within the time frame of the experiment at higher temperatures and higher ionic strength. Buffer conditions were 40 mM HEPES/KOH, pH 7.5, 5 mM MgCl₂, and 2 mM ATP with salt as indicated. Numbers represent the specific activities per active subunit (2 μM Hsp90α or hHsp90β) with or without the addition of 4 μM lidless constructs as indicated in the table. Observable stimulation is indicated by boldface type within the table.

| hHsp90α  | hHsp90β  |
|----------|----------|
| Alone    | With lidless hHsp90α | With lidless hHsp90β |
| 37 °C, 20 mM KCl | 0.05       | 0.055     |
| 37 °C, 150 mM KCl | 0.06       | 0.17      |
| 45 °C, 20 mM KCl | 0.12       | 0.24      |
| 45 °C, 150 mM KCl | 0.15       | 0.35      |

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**μM ATP/(min × μM Hsp90)**

| 37 °C, 20 mM KCl | 0.05       | 0.055 |
| 37 °C, 150 mM KCl | 0.06       | 0.17  |
| 45 °C, 20 mM KCl | 0.12       | 0.24  |
| 45 °C, 150 mM KCl | 0.15       | 0.35  |

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**μM ATP/(min × μM Hsp90)**

| 37 °C, 20 mM KCl | 0.06       | 0.065 |
| 37 °C, 150 mM KCl | 0.07       | 0.08  |
| 45 °C, 20 mM KCl | 0.13       | 0.61  |
| 45 °C, 150 mM KCl | 0.19       | 0.68  |
significantly smaller, implying that in particular the reaction responsible for trapping the ATP leads to the reduced ATP turnover of human Hsp90 versus yeast Hsp90.

Regarding differences between hHsp90α and hHsp90β, we observed that the exchange rates of the C-terminal dimerization site are very different between the two human and yeast Hsp90s, hinting at significant differences in the dimerization domains and the dimerization properties. hHsp90α appears to be less dynamic in this respect than hHsp90β, a feature that had been reported previously by Minami et al. (8, 35) as a failure to form hHsp90α monomers. Although our data show that dissociation of hHsp90α is possible but strongly disfavored over hHsp90β at standard conditions, these observations further suggest the possibility of developing isoform-specific inhibitors of ATP hydrolysis by interfering with Hsp90 dimerization. It is reasonable to assume that Hsp90α and Hsp90β have evolved to serve defined and independent cellular functions. The nature of these functions is just beginning to be understood for any pair of Hsp90s (38, 39).

In summary, our results show that the N-terminal dimerization reaction of human Hsp90 is similar to that of the cytosolic yeast Hsp90, the endoplasmic Grp94 (25), and the mitochondrial TRAP1 (40) protein, and therefore the functional coupling between ATP turnover and conformational changes is probably conserved among eukaryotic Hsp90 proteins. The basis for the low ATPase activity resides in higher barriers to the formation of the hydrolysis-competent N-terminal dimerized state.

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