Intrinsic Inhibition of the Hsp90 ATPase activity*
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The molecular chaperone Hsp90 is required for the folding and activation of a large number of substrate proteins. These are involved in essential cellular processes ranging from signal transduction to viral replication. For the activation of its substrates, Hsp90 binds and hydrolyses ATP, which is the key driving force for conformational conversions within the dimeric chaperone. Dimerization of Hsp90 is mediated by a C-terminal dimerization site. In addition, there is a transient ATP-induced dimerization of the two N-terminal ATP-binding domains. The resulting ring-like structure is thought to be the ATPase-active conformation. Hsp90 is a slow ATPase with a turnover number of 1 ATP per minute for the yeast protein. A key question for understanding the molecular mechanism of Hsp90 is how ATP hydrolysis is regulated and linked to conformational changes. In this study, we analyzed the activation process structurally and biochemically with a view to identify the conformational limitations of the ATPase reaction cycle. We show that the first 24 amino acids stabilize the N-terminal domain in a rigid state. Their removal confers flexibility specifically to the region between amino acid 98 and 120. Surprisingly, the deletion of this structure results in the complete loss of ATPase activity and in increased N-terminal dimerization. Complementation assays using heterodimeric Hsp90 show that this rigid lid acts as an intrinsic kinetic inhibitor of the Hsp90 ATPase cycle preventing N-terminal dimerization in the ground state. On the other hand, this structure acts – in concert with the 24 N-terminal amino acids of the other N-terminal domain – to form an activated ATPase and thus regulates the turnover number of Hsp90.
The molecular chaperone Hsp90 is required for the activation of more than 100 key cellular proteins, like kinases and transcription factors, such as steroid hormone receptors and the tumor suppressor protein p53 (1-4). Hsp90 represents about 1% of the cellular protein in eukaryotic cells and its expression levels further increase after exposure to cellular stressors (5). The ATPase-activity of Hsp90 is required for its essential cellular functions (6, 7). The importance of ATP hydrolysis for the function of Hsp90 is highlighted by the natural inhibitors geldanamycin and radicicol, which specifically target Hsp90 (8-10) and block the activation of substrate proteins by preventing ATP-binding to Hsp90 (11, 12).

ATP-hydrolysis by Hsp90 is a complex reaction that depends on the dimeric nature of the protein, which is preserved by a C-terminal dimerization domain (3, 13-15). Attempts to elucidate the mechanistic aspects of the ATPase reaction have resulted in the definition of a cyclic reaction pathway that leads to ATP-hydrolysis. ATP-binding induces conformational changes which change the surface properties of Hsp90 (16, 17). Subsequent investigations showed that contact formation between the two N-terminal domains of the dimeric protein results in a key conformation during the process of ATP-hydrolysis (13, 18). This conformation apparently is the result of domain rearrangements after the binding of ATP to the N-terminal binding sites. The rearrangements trap the ATP-molecule prior to hydrolysis (18) and lead to a dimeric state of the N-terminal domains (3). Biochemical studies indicate that the slow hydrolysis rate of Hsp90 (1 ATP/min for yeast Hsp90 (7, 19) and 0.05 ATP/min for human Hsp90 (20)) is the result of a slow formation of the N-terminally dimerized state. This is – among other indications – based on the finding, that under steady-state ATP-hydrolysing conditions only a very low population of wt-Hsp90 can be found in the N-terminally dimerized conformation, which indicates that reactions prior to its formation are rate-limiting (3, 21, 22). The first 24 amino acids of the N-terminal domain are important for this dimerization reaction. Their deletion results in a mutant, which is unable to hydrolyze ATP and unable to form productive N-terminal dimers (21). For Hsp90, no structural data are available that show how ATP hydrolysis is achieved in atomic detail. Structural homology of the N-terminal ATP-binding site defines a family of proteins termed GHKL-ATPases, which are suspected to share a similar ATPase mechanism (23). In the case of the three homologous proteins, Grp94, GyraseB and MutL, crystal structures of N-terminal dimerized conformations that could resemble the ATPase-active state have been reported even in fragments, lacking the C-terminal dimerization site (24-27). In all three structures of crystallized GHKL-ATPases the first helix is repositioned to provide an interaction site for the N-terminal dimerization. In MutL and GyraseB – in addition – the first strand of the beta-sheet is exchanged between the two N-terminal domains forming an N-terminally dimerized state. This conformation depends on the presence of the non-hydrolysable ATP-analogue AMP-PNP, as in the absence of nucleotides this contact formation is not observed (28, 29). The structure of the nucleotide-binding domain of these three proteins is very similar to that of Hsp90 regarding the seven stranded E-sheet and the positioning of the α-helices (12, 23, 30). Key structural differences between Hsp90 and the GHKL-ATPases MutL and GyraseB involve a region termed the “ATP-lid”, which consists of two α-helices that close the ATP-binding pocket in the crystal structures of N-terminal dimerized GHKL-ATPases. Despite the availability of some crystal structures, the mechanistic aspects of the ATPase reaction are not understood for any of the GHKL-ATPases. As such, the reasons for the slow ATP turnover and many aspects of the conformational control of the hydrolysis reaction remain unknown, although they
are at the heart of the chaperone mechanism of Hsp90. It is therefore of utmost importance for understanding the mechanism of the molecular chaperone Hsp90 to define the conformational dynamics that govern the ATPase cycle. Here we show by high resolution structure analysis and biochemical approaches that the pace of Hsp90 is regulated by a well-defined inhibitory element.

MATERIALS AND METHODS

Materials – 13C6-Glucose was obtained from Cambridge Isotope Laboratories, Andover, USA and 15NH4Cl was obtained from Spectra Stable Isotopes, Columbia, MD, USA. Radicicol was from Sigma, St. Louis, USA. Geldanamycin was a kind gift of the NCI Experimental Drug Division, National Institutes of Health (Bethesda, MD). All other chemicals were obtained from Merck, Darmstadt, Germany.

Cloning and Protein Purification – The lidless-mutant of yeast Hsp90 was generated using standard molecular biology techniques. The amino acids 98 to 121 were deleted and replaced by a SerGlySer-linker. Fragments N-terminal and C-terminal to the site of the mutation were amplified and an overlap containing the SerGlySer-linker was generated by designing the according primers (5’-GGTACCAGTGCAAAGGC-GGTAGCCTTGGTTTTTACTCTTTATTCTAGTTG-3’ and 5’-GTAAAAACCAACGCTACCGCTCTTG-GCAATGGTACCCAAGTTATTAATC-3’). The final fragment was generated by performing a linker-PCR using the two fragments as templates. The construct was then inserted into the pET28a-vector (Novagen, Madison, WI) for protein expression using the NdeI/BamHI restriction sites. The truncated versions of this mutation (lidless-N210, lidless-N530, Δ24-lidless-Hsp90) were constructed using this plasmid as a template. All other proteins used in the study were derived from yeast Hsp90 as well. All proteins were expressed in the E. coli strain BL21 (DE3) RIL. Bacterial cultures were grown to an OD600 of 0.8 and induced with 1.0 mM IPTG. The proteins were purified according to the published protocol (3). All proteins, except lidless-Hsp90, were stable and could be stored at –80 °C in standard buffer (40 mM HEPES, 20 mM KCl, pH 7.5). Lidless-Hsp90 showed a tendency to form aggregates at high protein concentrations and therefore it was concentrated only to 2 mg/ml and stored in a buffer containing 40 mM HEPES, 300 mM KCl, pH 7.5. The purity of all proteins was assessed by SDS-PAGE and determined to be higher than 95%. MALDI-TOF mass spectroscopy was used to determine the molecular mass and the integrity of the purified proteins. The extinction coefficients to calculate the protein concentration were obtained from the ProtParam-analysis of the primary sequences. (http://us.expasy.org/tools/protparam.html)

Crystallization and Structure Determination – For crystallization, 2 μl of a solution containing 10 mg/ml Δ24-N210 in 40 mM HEPES, 20 mM KCl, pH 7.5 were mixed with 2 μl of a reservoir solution consisting of 0.2 M magnesium acetate, 0.1 M sodium cacodylate pH 6.5 and 20% PEG8000 as precipitant. Monoclinic crystals of space group C2 appeared after four days. The crystals were transferred to the mother solution containing 20% glycerol as cryoprotectant, and were mounted under a nitrogen stream (100 K) on a MAR345 image plate system (MAR Research, Hamburg). A complete X-ray data set to a maximum resolution of 1.94 Å was taken from a single crystal. The data were processed with MOSFLM, and scaled and loaded using SCALA from the CCP4 program suite (http://www.ccp4.ac.uk/). After rigid body refinement with CNS using the coordinates of the tetragonal form of the N-terminal domain of yeast Hsp90 (1AH6 (31)), the
R-factor dropped to 36%. The crystallographic refinement was done in several cycles consisting of model building steps performed with MAIN (http://www-bmb.ijs.si/) and conjugate gradient minimization and simulated annealing using CNS (http://cns.csb.yale.edu/v1.1/). Finally, solvent molecules were built in and individual, restrained atomic B values were refined. The occupancy of atoms not defined by electron density was set to zero. All main chain angles are located in the most favored and favored regions of the Ramachandran plot. The atomic coordinates have been deposited with the protein data base (PDB; http://www.rcsb.org/pdb/) under the code 2AKP.

Protein Labeling and NMR Backbone Resonance Assignment – For NMR experiments, Δ24-N210 was purified enriched with $^{13}$C, $^{15}$N. Bacteria were grown in LB Amp to a density of 0.2. After harvesting by centrifugation and washing with M9-medium containing 2g/l $^{13}$C-$\alpha$-Glucose and 1g/l $^{15}$NH$_4$Cl, bacteria were grown until the OD reached 0.4 and protein expression was induced by adding IPTG to a final concentration of 1.5 mM. Expression was performed at 30 °C for 15 hours. Bacteria were harvested thereafter and protein purification was performed according to the standard protocol (19). The purified protein was dialyzed against 10 mM KPO$_4$, pH 7.5 and concentrated to 15 mg/ml (0.6 mM). After addition of 10% D$_2$O the sample was used for NMR measurements.

For assignment of $^1$H, $^{15}$N, $^{13}$C$\alpha$, and $^{13}$CB, a set of triple resonance experiments consisting of HNCO, HN(CA)CO, HNCA, HN(CO)CA, HNCA CB, HN(CO)CA CB (32) was recorded at 30°C on Bruker DMX 600, 750 and Avance 900 spectrometers. $^1$H, $^{15}$N HSQC, $^{15}$N edited 3D NOESY-HSQC and HSQC-NOESY-HSQC (80ms mixing time) (33) were recorded using a $^{15}$N labeled sample. Spectra were processed using XWINNMR3.6 (Bruker, Rheinstetten) and analyzed using SPARKY (34). Sequence-specific resonance assignments were obtained with the assignment program PASTA (35).

$^{15}$N backbone relaxation – $^{15}$N relaxation measurements ($R_1$, $R_2$, $^{15}$N{H}heteronuclear NOE) were performed at 30°C and 750 MHz using an 800μM $^{15}$N labeled sample. For the $R_1$ measurement eleven different mixing times and for the $R_2$ measurement eight different mixing times were used with three duplicate points for error estimation. The pulse schemes used were modifications of experiments described earlier (36). A first initial guess of the molecular rotational diffusion tensor was obtained from the $R_2/R_1$ ratios of the individual residues using the programs R2R1_TMX (A.G. Palmer III, Columbia University) and QUADRATIC_DIFFUSION (A.G. Palmer III, Columbia University) and PDB coordinate files 1AH6 (N210) and the coordinate file of Δ24-N210 as determined in this study, respectively. Highly mobile residues or residues with relaxation contribution from chemical exchange were excluded from the estimation. Model-free analysis of the relaxation data was performed using MODELFREE4.1 (A.G. Palmer III, Columbia University) interfaced with FAST-MODELFREE (37). Rigid body hydrodynamic calculations of the diffusion tensor were performed with the program HYDRONMR (38) using the same coordinates as for the MODELFREE analysis.

Protein stability measurements – CD-spectroscopy was performed using a Jasco 715 spectropolarimeter (Jasco, Groß-Umstadt, Germany). Protein samples were diluted to 0.1 mg/ml in 10 mM KH$_2$PO$_4$/K$_2$HPO$_4$, pH 7.4 and measurements were performed in a quartz cuvette with a path length of 0.1 cm. All spectra were corrected for buffer background by subtracting pure buffer spectrum. Quantitative deconvolution of the CD-spectra was performed with the software CDNN, which uses a learning
Determination of the quaternary structure – Determination of the quaternary structure of Hsp90 and variants thereof was performed by size-exclusion HPLC on a Superdex 200HR column (Amersham Biosciences Europe, Freiburg, Germany) equilibrated in 40 mM HEPES, 150 mM KCl, pH 7.5. Samples were loaded at different concentrations in a volume of 100 μl and separated at 0.5 ml/min. Proteins eluting from the column were detected by intrinsic fluorescence at an excitation wavelength of 280 nm. It had been reported for Hsp90 previously that the elution time shows a concentration-dependent shift, which can be used to calculate a approximate dimerization constant in the range of 60 to 100 nM (3). To perform this analysis, the data points were fitted using SIGMAPLOT as previously described (3, 41).

Nucleotide binding kinetics – Stopped-flow measurements were performed with a HiTech SF-61 DX2 instrument (Hi-Tech, Salisbury, UK) in 40 mM HEPES pH 7.5, 20 mM KCl, 5 mM MgCl₂. The excitation slit was set to 0.5 nm, the excitation wavelength to 296 nm (Hg-line) for intrinsic tryptophan/(Pγ)-MABA-ATP energy transfer (FRET) and the emission signal was detected through a long pass glass-filter with 400 nm cut-off. The temperature was set to 25°C. Dissociation rate constants were measured directly by displacement of preformed Hsp90-(Pγ)MABA-ATP complexes with excess unlabelled ligand. The observed kinetic could be analyzed by using single exponential equations. Association rate constants were derived from a series of experiments where the concentration of (Pγ)MABA-ATP was varied, while the concentration of protein was left unchanged. Data analysis was performed essentially as described (21).

Hsp90 in vivo rescue assay - Hsp90 functionality in vivo was investigated using a plasmid shuffling approach based on the system described previously (43). Lidless-
Hsp90 and wt-Hsp90 were cloned into the p2HGal-vector and transformed into a yeast strain which lacks the genomic HSP82 and HSC82 and instead carries a plasmid coding for HSP82 under the control of a constitutive GPD-promotor (43). This plasmid also contains an URA selection marker, which can be counter selected by addition of 5’FOA to the growth medium. Loss of this plasmid is required to prevent lethal toxicity of 5’FOA and only if the supplied Hsp90-variant on the p2HGal-plasmid is functional, growth of the yeast strain will be observed.

RESULTS

Removal of the N-terminal amino acids of Hsp90 reveals a segment with unusual flexibility – The ATP binding site of Hsp90 is located in the N-terminal domain. The ATPase activity depends on conformational changes after ATP binding which lead to N-terminal dimerization (3, 13). The Hsp90-mutant Δ24-Hsp90, which lacks the first 24 amino acids, is inactive in ATP hydrolysis (21). In addition, after forming a heterodimer with a wt-Hsp90 subunit, the ATP-hydrolysis activity of this assembly is dramatically reduced compared to the wt-dimer, indicating that Δ24-Hsp90 is unable to induce the trans-activation required to stimulate the ATPase activity of a wt-Hsp90 subunit in a heterodimer (21). This implies that critical residues involved in the N-terminal dimerization reaction are either missing or are modified in their structure and thus can no longer perform their tasks. The deletion of the first 24 amino acids possibly mimics an intermediate during the dimerization process, given the current model of N-terminal strand exchange between the two ATP-binding sites (21, 25, 27). The removal of the first 24 amino acids had no effect on the global stability of the N-terminal domain (21). The positioning of these amino acids in the wt-protein allows their removal, as they are sited at the periphery of the structure and the α-helix forms only few contacts to the accompanying α-helix (amino acids 101-110), but does not appear to contribute to the overall stability of the β-sheet.

To investigate which structural changes occurred as a result of the N-terminal deletion, we crystallized Δ24-N210 and solved its structure. The crystals diffracted to 1.94 Å and the structure was solved by molecular replacement (parameters in Table 1), using the structure of the wt N-terminal domain of yeast Hsp90 as a template (31). The two proteins were super imposable in the area of the β-sheet and most of the helices were also not affected by the deletion of the first 24 amino acids (Figure 1). Striking differences were observed for amino acids 90 to 130, where the structure was largely distorted and the position of the two helices was changed. The new conformation of the helices represents a more open position of the binding pocket, as the helices were moved outside toward the position that is normally occupied by the first 24 amino acids. The distortion of this ATP-lid is an interesting finding, as it highlights the importance of the contacts between the first alpha-helix and the ATP-lid in the wt-protein. These structures stabilize each other in the ground state of the N-terminal domain. In addition, it suggests flexibility of the ATP-lid once it is released from its contacts with the N-terminal domain.

To compare the dynamic properties of the Δ24-N210 mutant with wt-N210, we used NMR relaxation measurements. For wt-N210, about 192 of the 210 amino acids had been unambiguously assigned previously (44, 45). For Δ24-N210, 163 of its 186 amino acids could be assigned in this study (Figure 2), which was additionally confirmed by sequential N-H, N-H and H-N-H NOE contacts. These NOE contacts agree well with the crystal structure, showing that no overall structural rearrangements occurred between soluble state and crystallized form (data not shown). The dynamics of the backbone structures were investigated by
15N relaxation measurements. Prior to motional analysis of the backbone 15N relaxation data, an estimation of the rotational diffusion tensor of N210 and Δ24-N210 was performed (Table 2). Therein, D is indicative of the size and shape of the molecule, $\chi^2$ is a measure for the goodness of the fit to experimental $R_2/R_1$ ratios assuming a particular diffusional model and the F-test value (F) judges if a more complicated model is statistically satisfied. For both proteins, the axially symmetric diffusion model was chosen based on Monte Carlo simulations and F-Testing. Rotational correlation times obtained from hydrodynamic calculations using the structure of the monomeric proteins correlate well with the estimated correlation times (Table 2), indicating that even at high protein concentrations used for NMR, both proteins are monomeric. Based on these data, a Modelfree analysis resulted in the squared order parameters $S^2$, which are indicative for motional restriction. For N210, an equal distribution of the squared order parameter $S^2$ around an average value of 0.93 indicated regions with significantly increased mobility were not present (Figure 3A). In contrast, after deletion of the N-terminal strand, a significant increase in the flexibility of amino acids 90 throughout 120 is observed, while the dynamics of the remainder of the protein do not appear to be altered (average $S^2$ of 0.93) (Figure 3B). This stretch of amino acids corresponds exactly to the distorted region observed in the crystal structure. Aligning the dynamic region with the sequence of other GHKL-ATPases shows significant sequence homology within the presumed lid-region (Figure 3C).

Interestingly, the corresponding region is known to be dynamic in the crystal structures of MutL (24), GyraseB (46) and HtpG (47). Therefore it is likely that the intimate contact formation between helix 1 and the ATP-lid in yeast Hsp90 leads to a more static ground state, which precludes dynamic behavior.

The deletion of the ATP-lid does not affect nucleotide-binding to Hsp90 – To further investigate the contribution of the ATP-lid to the ATPase-cycle, we deleted the region from amino acids 98 to 121 and replaced this loop structure with a SerGlySer-linker (lidless-Hsp90). Based on the structure, this linker should be sufficient to reconnect the peptide chain after removal of the ATP-lid as indicated in Figure 1B. As a deletion within a domain may result in global distortion of the domain, we first investigated the structure and the stability of the isolated N-terminal domain after deletion of the ATP-lid (lidless-N210). The secondary structure analysis performed by CD-spectroscopy indicated a reduction in alpha-helical content from about 40% to 22% (Figure 4A). This corresponds to the loss of the helices of the ATP-lid. The stability of the variant was investigated by urea transitions (Figure 4B). The comparison of the stability of lidless-N210 with that of the wt-domain showed that the cooperativity of the unfolding transition is preserved, although the stability is slightly reduced with a transition midpoint at 4.1 M instead of 4.6 M urea. Thus, the structural integrity of the N-terminal domain is not distorted by deletion of the ATP-lid.

To analyze the influence of the lid on the nucleotide-binding properties, we determined the association and dissociation rate constants for MABA-ATP by stopped-flow fluorescence spectroscopy. MABA-ATP changes its fluorescence by binding to Hsp90 and leads to a FRET-signal that arises from Foerster transfer between the MABA-label and an intrinsic tryptophan of the protein (18). To address whether nucleotide-binding is affected by the deletion of the ATP-lid, we compared the isolated N-terminal domains in their lidless and wt-forms with respect to their association and dissociation rates for MABA-ATP. Nucleotide binding did not change significantly for lidless-N210 compared to wt-N210 or wt-Hsp90 (Table 3). Neither did we observe changes in the binding properties of the full-length protein after deletion of the ATP-lid. However,
when we deleted both the first 24 amino acids and the ATP-lid (Δ24-lidless-Hsp90), we observed a seven fold higher dissociation rate for MABA-ATP (Figure 4C) and a four fold higher association rate constant (Table 3). This suggests that exchange of the nucleotide between binding pocket and solvent is less hindered after removal of the helical structures in this mutant.

The ATP-lid inhibits the catalytic efficiency – The contribution of the ATP-lid to the ATPase activity of Hsp90 is unknown. In the structure of the N-terminal domain, no contacts between the ATP-lid and the ATP-binding site can be seen (30). We performed ATPase assays of lidless-Hsp90 and discovered that this mutant lacks ATPase activity (data not shown). In addition, we found this mutant to be insufficient to rescue a HSP82Δ/HSC82Δ yeast strain (data not shown), implying impaired in vivo function. The apparent complexity of the ATPase cycle of Hsp90 precludes immediate conclusions about the step inhibited by the mutation.

Previous studies had shown that heterodimeric Hsp90 constructs allow studying the conformational changes that involve interactions between the two subunits of the Hsp90-dimer (3). It is well established that Hsp90 mutants containing the C-terminal dimerization site form heterodimers with the wt-protein (3). For lidless-Hsp90, it seemed possible – based on the known structures of GHKL-family members – that the deletion of the ATP-lid specifically prevented N-terminal dimerization, as it may form contacts with the intruding strand of the other subunit (24, 26, 27). To address this, we formed heterodimers of lidless-Hsp90 with wt-Hsp90 and determined the turnover number of wt-Hsp90 in the presence of different concentrations of lidless-Hsp90, while leaving the wt-Hsp90 concentration constant at 2 μM. Unexpectedly, in this experiment, a marked increase in turnover was found in the presence of lidless-Hsp90. Due to the absence of ATPase-

activity in lidless-Hsp90, this increase is a consequence of heterodimer formation. To obtain the activity of the heterodimer, the saturation curve was analyzed (Figure 5). The turnover of wt-Hsp90 was increased 9fold, if the interacting partner on the other side did not contain the ATP-lid. Similar experiments were performed with previously characterized Hsp90 mutants. We did not obtain any gain of activity for the ATPase-inactive point mutants E33A-Hsp90 and D79N-Hsp90 (6,7) (Table 4). These mutants were found previously to be sufficient to maintain hydrolysis activity in heterodimers with wt-Hsp90, although they lack intrinsic ATPase activity (3). Therefore, the strong increase observed with the addition of lidless-Hsp90 appears not to be explained by gained activity of the lidless-Hsp90 subunit in the heterodimer with wt-Hsp90. In consequence, the activation of hydrolysis occurs exclusively in the wt-Hsp90 subunit, indicating that lidless-Hsp90 serves as a potent stimulation partner.

We set out to investigate other mutations with known modifications in the N-terminal dimerization reaction. As such, Δ8-Hsp90 had been shown to hydrolyze ATP normally, although it forms stronger N-terminal dimers than wt-Hsp90 in the presence of ATP (21). Δ16-Hsp90, in contrast, does not hydrolyze ATP (21). The combination of these mutants with lidless-Hsp90 showed that Δ8-Hsp90 is strongly stimulated (about 6 to 7fold) in heterodimers with lidless-Hsp90 and even Δ16-Hsp90 is stimulated to an activity of about 1 ATP/min, which roughly corresponds to the turnover of homodimeric wt-Hsp90 (Table 4). Thus, the deletion of the ATP-lid results in a protein, which serves as a better platform for the stimulation of the wt-Hsp90 ATPase activity, albeit being hydrolysis-incompetent itself.

Deletion of the ATP-lid leads to stable N-terminal dimerization – N-terminal dimerization is critical during the process of ATP turnover by yeast Hsp90 (3, 13).
Biochemical evidence suggests that ATP-hydrolysis occurs in the dimerized conformation (22), and the events leading to this form are rate-limiting (13, 21). Thus any detectable influence on the turnover number is potentially the result of changed N-terminal dimerization properties.

To address more directly whether the N-terminal dimerization properties were affected by deleting the ATP-lid, we performed size exclusion chromatography as described previously (3). Different concentrations of the individual Hsp90-constructs were applied to an analytical gel filtration column and the elution time of the peak was determined. A decrease in the concentration of Hsp90 results in a progressive increase of retention time from 20 minutes to 23 minutes. This methodology had been used to estimate the dimerization constant of about 60 nM for Hsp90 (3). In addition, it was used to prove that the major contribution to the overall dimerization reaction comes from the C-terminal interaction site, while the participation of the N-termini is undetectable in wt-Hsp90 (3, 14). We performed this experiment with wt-Hsp90 and lidless-Hsp90. Wt-Hsp90 showed the gradual shift in retention time (Figure 6A), as reported earlier (3). Lidless-Hsp90, in sharp contrast, did not dissociate as is evident from the stable retention time (Figure 6B), even at concentrations where the detection of the peak becomes difficult (35 nM applied protein). These data show that by removing the ATP-lid, it is possible to trap Hsp90 in a stable N-terminal dimerized conformation.

The ATP-lid and the first 24 amino acids cooperate to form N-terminal dimers – It had been proposed previously that the first 24 amino acids are important for the N-terminal dimerization reaction (21). The results presented above suggest that the repositioning of the ATP lid is a key step for the regulation of the ATPase cycle. In the absence of the lid, increased N-terminal dimerization is observed and heterodimeric constructs with a wt-Hsp90 subunit show increased cycling rates. To confirm the involvement of the N-terminal strand in this reaction, we constructed a mutant in which the first 24 amino acids from Hsp90 were deleted in addition to the ATP-lid. This mutant was supposed to be unable to form stable N-terminal dimers, if the first 24 amino acids were directly involved in the N-terminal dimerization process. The resulting protein was stably folded as judged by a similar thermal stability compared to the wt-protein (data not shown). We performed size exclusion chromatography experiments with this mutant to analyze the dimerization properties. In contrast to lidless-Hsp90, a shift of the peak to longer retention times can be obtained at lower protein concentrations (Figure 6C). This shows that formation of dimers is strongly reduced by this additional mutation and the dimerization affinity is in the range of wt-Hsp90 again, which is mostly resulting from the C-terminal dimerization site (3). Therefore we conclude that, while the deletion of the ATP-lid exposes the N-terminal dimerization site and leads to increased formation of N-terminal dimers, the additional deletion of the first 24 amino acids reverses this effect.

To test the effect of the double deletion on ATPase activity, we used the ATPase-inactive lidless-Δ24-Hsp90 mutant and formed heterodimers with wt-Hsp90. As in the experiments with Δ24-Hsp90 (21), this mutant acted as an inhibitor of ATP-hydrolysis and thus appears unable to form productive N-terminal dimers (Figure 7). Taken together, it is evident that deletion of the ATP-lid leads to an Hsp90-mutant that greatly favors the N-terminal dimerization reaction, while the additional deletion of the first 24 amino acids eliminates the ability to form N-terminal dimers.
DISCUSSION

The ATPase cycle of Hsp90 couples the energy of ATP binding and hydrolysis to a series of structural changes within the dimeric protein, which are thought to induce or maintain a certain conformation of a substrate protein (48, 49). Biochemical investigations of the ATPase cycle revealed a controlled order of steps which are required for the hydrolysis of ATP (3, 13, 18). Key steps are a transient N-terminal dimerization and the association of the N-terminal domain with the middle domain. Hydrolysis of the ATP-molecule is thought to be mediated by this conformation and the events leading to this state seem to be the rate-limiting conformational changes during the ATPase cycle, limiting the ATPase activity of yeast Hsp90 to 1/min (7) and human Hsp90 to 0.05/min (20). The mechanistic reason for the low ATPase-activity of Hsp90 has not been identified yet. Puzzling as well was the fact that no part of the N-terminal domain of Hsp90 appeared flexible enough to allow the conformational changes required to form a dimeric state, in analogy to the ones observed for MutL (24) and GyraseB (27), which together with Hsp90 form the class of homologous GHKL-ATPases. The crystal structures of the N-terminal domains of human and yeast Hsp90 are monomeric, irrespective of the presence of ATP. However, the crystal structure of the ER-based homologue of Hsp90, Grp94, in complex with nucleotides showed an N-terminally dimerized conformation, which resembles in parts the ones observed for other GHKL-ATPases (25). In contrast, the recent structure of the N-terminal and middle domain of prokaryotic Hsp90 showed monomeric subunit organization (47).

The first 24 amino acids of yeast Hsp90 have been implicated in the process of N-terminal dimerization by biochemical approaches (21), which would be in agreement with the crystal structures of the homologous proteins MutL and GyraseB (24, 27). In these structures, the corresponding stretch of amino acids is used to make contacts between the two N-terminal domains. We therefore investigated the effects of removing the first 24 amino acids of yeast Hsp90 on the structure and dynamics of the N-terminal domain by crystal structure determination and 3D-NMR and obtained a striking difference to the wt-protein. Upon removal of this stretch, the amino acids corresponding to the ATP-lid gained flexibility. Apparently, these two regions of the N-terminal domain, the first 24 amino acids and the ATP-lid, stabilize each other in a rather rigid ground-state. The stabilization seems to be mediated by one helix of the ATP-lid (amino acids 101-110) and the α-helix within the first 24 amino acids, composed of amino acids 9 to 24. In the crystal structure of the N-terminal domain of Hsp90 (N210), these two helices are in close vicinity in the ground-state and form several contacts with each other (30). The importance of this interaction for the process of ATP hydrolysis becomes also evident from single point mutations that have been identified in this region. Two of the mutations that influence ATP-turnover, T101I and D107N, are found directly within the ATP-lid, another mutation T22I is within the first helix (43, 50). These mutations also affect the binding affinities for the partner proteins Sba1 and Aha1 to Hsp90 (50), which were also shown to be dependent on the conformational state of the Hsp90-dimer (13, 22, 50-56). In addition, for Hsp90, the ATP-lid serves as the binding site for the co-chaperone Cdc37 (57), which inhibits the ATPase-activity of Hsp90.

The role of the ATP-lid for the process of contact formation between the N-terminal domains was unknown so far and structural data regarding its involvement in dimerization and hydrolysis are confusing. In an N-terminally truncated Grp94, this region is in a more open state, once the AMP-PNP induced dimers are formed (25). In MutL and GyraseB, in contrast, the ATP-lid is
folded over the ATP-binding site in the dimerized conformation. Our results clearly show that the ATP-lid is not needed for the N-terminal dimerization. Instead, our results suggest a novel role of the ATP lid which explains the slow ATPase turnover of yeast Hsp90: the ATP lid is an intrinsic inhibitor of the ATP hydrolysis reaction, as it keeps the dimerization site inactive in its ground state (Figure 8). Upon removal of the ATP-lid, the ability of the N-terminal domain to dimerize increases dramatically. Furthermore, the variant lacking this lid is in a stable N-terminally dimerized conformation. Size-exclusion chromatography indicates at least a 50-fold gain in dimerization affinity. The increased dimerization goes along with increased stimulatory effects on the ATPase of the accompanying subunit in the heterodimer. This indicates that with the exposure of the N-terminal dimerization site, the rate-limiting step of the ATPase-reaction – the conformational changes leading to N-terminal dimerization – has been accelerated at least 9fold.

On the other hand, the ATP-lid serves a second function, which is evident from the inability of lidless-Hsp90 homodimers to hydrolyze ATP. Even if heterodimers with strongly trans-stimulating, but inactive Hsp90-variants are formed (e.g. E33A-Hsp90), no hydrolysis of ATP can be observed after deletion of the ATP-lid. In addition, our data show that the ATPase within the lidless-subunit is not functional in the absence of the ATP-lid. These data suggest that the removal of the ATP-lid from the ground state allows the N-terminal dimerization reaction to occur, while the positioning of the ATP-lid in its new orientation is required for the ATP hydrolyzing reaction in this domain. ATP-hydrolysis within lidless-Hsp90 is blocked in the N-terminally dimerized conformation; the binding kinetics and energetics for nucleotide binding are not substantially affected. These data suggest that regions within the lid or controlled by the ATP-lid are involved in the hydrolysis reaction in this subunit in addition to the described function of inhibiting N-terminal dimerization. This kind of coupling would ensure that catalytic centers only obtain activity in an N-terminally dimerized conformation of Hsp90, thereby eliminating inefficient cycling of Hsp90 in the absence of conformational movements.

These data show that sophisticated conformational changes in the nucleotide-binding domains of Hsp90 and potentially other GHKL-ATPases regulate the hydrolysis of the bound ATP. The flexibility of the helices 1 (amino acids 9 to 24), 4 and 5 (ATP-lid) governs to a large extent the pace of the ATPase reaction and may be the cause for the differences in the orientation of the ATP-lid in MutL, GyraseB, HtpG, Grp94 and Hsp90. The rather rigid ground state of Hsp90 within the lid-region sets Hsp90 apart from other GHKL-ATPases, while the concepts of the ATPase cycle and the regions participating in its control might be preserved.
FOOTNOTES

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The Abbreviations used are:
AMP-PNP, 5’-Adenylimido-diphosphate, HSQC, heteronuclear single quantum coherence, Δ8-Hsp90, deletion mutant lacking the first 8 amino acids; Δ16-Hsp90, deletion mutant lacking the first 16 amino acids; Δ24-Hsp90, deletion mutant lacking the first 24 amino acids; Δ24-N210, the N-terminal domain of yeast Hsp90 (amino acid 1-210) lacking the first 24 amino acids; FOA, 5’-fluor orotic acid; HtpG, Hsp90 from E.coli; HSC82, the S. cerevisiae gene for Hsc90; HSP82, the S. cerevisiae gene for Hsp90; Hsp90, heat shock protein 90; 262C-Hsp90, yeast Hsp90 fragment ranging from amino acid 262 to 709; IPTG, isopropyl-β-D-thiogalactoside; lidless-Hsp90; yeast Hsp90 with deletion of amino acids 98 to 121; Δ24-lidless-Hsp90, yeast Hsp90 after deletion of the ATP-lid (amino acids 98 to 121) and the first strand (amino acids 1 to 24); lidless-N210; N-terminal domain with deletion of amino acids 98 to 121; lidless-N530; N-terminal and middle domain with deletion of amino acids 98 to 121; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; (Pγ)MABA-ATP, adenosinetriphospho-γ-(N’-methylanthraniloylaminobutyl)-phosphor-amidate; SDS, sodium-dodecylsulfate; wt, wild type; wt-N210, N-terminal domain of Hsp90, consisting of amino acids 1 to 210; wt-N530, N-terminal and middle domain of Hsp90, consisting of the amino acids 1 to 530.

REFERENCES

1. Picard, D. (2002) Cell Mol Life Sci 59, 1640-8
2. Pratt, W.B. & Toft, D.O. (2003) Exp Biol Med (Maywood) 228, 111-33
3. Richter, K., Muschler, P., Hainzl, O. & Buchner, J. (2001) J Biol Chem 276, 33689-96
4. Prodromou, C. & Pearl, L.H. (2003) Curr Cancer Drug Targets 3, 301-23
5. Welch, W.J. & Feramisco, J.R. (1982) J Biol Chem 257, 14949-59
6. Obermann, W.M., Sondermann, H., Russo, A.A., Pavletich, N.P. & Hartl, F.U. (1998) J Cell Biol 143, 901-10
7. Panaretou, B., Prodromou, C., Roe, S.M., O’Brien, R., Ladbury, J.E., Piper, P.W. & Pearl, L.H. (1998) Embo J 17, 4829-36
8. Whitesell, L., Minnaugh, E.G., De Costa, B., Myers, C.E. & Neckers, L.M. (1994) Proc Natl Acad Sci U S A 91, 8324-8
9. Schulte, T.W., Akinaga, S., Soga, S., Sullivan, W., Stensgard, B., Toft, D. & Neckers, L.M. (1998) Cell Stress Chaperones 3, 100-8
10. Sharma, S.V., Agatsuma, T. & Nakano, H. (1998) Oncogene 16, 2639-45
11. Roe, S.M., Prodromou, C., O’Brien, R., Ladbury, J.E., Piper, P.W. & Pearl, L.H. (1999) J Med Chem 42, 260-6
12. Stebbins, C.E., Russo, A.A., Schneider, C., Rosen, N., Hartl, F.U. & Pavletich, N.P. (1997) Cell 89, 239-50
13. Prodromou, C., Panaretou, B., Chohan, S., Siligardi, G., O’Brien, R., Ladbury, J.E., Roe, S.M., Piper, P.W. & Pearl, L.H. (2000) Embo J 19, 4383-92
14. Minami, Y., Kimura, Y., Kawasaki, H., Suzuki, K. & Yahara, I. (1994) Mol Cell Biol 14, 1459-64
15. Wegele, H., Muschler, P., Bunck, M., Reinstein, J. & Buchner, J. (2003) J Biol Chem 278, 39303-10
16. Sullivan, W., Stenggard, B., Caucutt, G., Bartha, B., McMahon, N., Alnemri, E.S., Litwack, G. & Toft, D. (1997) J Biol Chem 272, 8007-12
17. Csermely, P., Kajtar, J., Hollosi, M., Jaloszvszky, G., Holly, S., Kahn, C.R., Gergely, P. Jr., Soti, C., Mihaly, K. & Somogyi, J. (1993) J Biol Chem 268, 1901-7
18. Weikl, T., Muschler, P., Richter, K., Veit, T., Reinstein, J., Buchner, J. (2000) J Mol Biol 303, 583-92
19. Scheibel, T., Weikl, T. & Buchner, J. (1998) Proc Natl Acad Sci U S A 95, 1495-9
20. McLaughlin, S.H., Smith, H.W. & Jackson, S.E. (2002) J Mol Biol 315, 787-98
21. Richter, K., Reinstein, J. & Buchner, J. (2002) J Biol Chem 277, 44905-10
22. Richter, K., Walter, S. & Buchner, J. (2004) J Mol Biol 342, 1403-13
23. Dutta, R. & Inouye, M. (2000) Trends Biochem Sci 25, 24-8
24. Ban, C., Junop, M. & Yang, W. (1999) Cell 97, 85-97
25. Immormino, R.M., Dollins, D.E., Shaffer, P.L., Soldano, K.L., Walker, M.A. & Gewirth, D.T. (2004) J Biol Chem 279, 46162-71
26. Brino, L., Urzhumtsev, A., Mousli, M., Bronner, C., Mitschler, A., Oudet, P. & Moras, D. (2000) J Biol Chem 275, 9468-75
27. Wigley, D.B., Davies, G.J., Dodson, E.J., Maxwell, A. & Dodson, G. (1991) Nature 351, 624-9
28. Ban, C. & Yang, W. (1998) Cell 95, 541-52
29. Dollins, D.E., Immormino, R.M. & Gewirth, D.T. (2005) J Biol Chem
30. Prodromou, C., Roe, S.M., O'Brien, R., Ladbury, J.E., Piper, P.W. & Pearl, L.H. (1997) Cell 90, 65-75
31. Prodromou, C., Roe, S.M., Piper, P.W. & Pearl, L.H. (1997) Nat Struct Biol 4, 477-82
32. Sattler, M., Schleucher, J., Griesinger, C. (1999) Prog. Nucl. Magn. Reson. Spectrosc. 34, 93-158
33. Jahnke, W., Bauer, C., Gemmecker, G., Kessler, H. (1995) J. Magn. Reson. Series B 106, 86-88
34. Goddard, T.D., Kneller, D.G. SPARKY 3. University of California, San Francisco.
35. Leutner, M., Gschwind, R.M., Liermann, J., Schwarz, C., Gemmecker, G. & Kessler, H. (1998) J Biomol NMR 11, 31-43
36. Farrow, N.A., Muhandiram, R., Singer, A.U., Pascal, S.M., Kay, C.M., Gish, G., Shoelson, S.E., Pawson, T., Forman-Kay, J.D., Kay, L.E. (1994) Biochemistry 33, 5984-6003
37. Cole, R. & Loria, J.P. (2003) J. Biomol. NMR 26, 203-213
38. de la Torre, J.G., Huertas, M.L. & Carrasco, B. (2000) J. Magn. Reson. 147, 138-146
39. Andrade, M.A., Chacon, P., Merelo, J.J. & Moran, F. (1993) Protein Eng 6, 383-90
40. Ali, J.A., Jackson, A.P., Howells, A.J. & Maxwell, A. (1993) Biochemistry 32, 2717-24
41. Richter, K., Meinlschmidt, B. & Buchner, J. (2005) in Protein Folding Handbook, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, New York, p768-829
42. Tochtrop, G.P., Richter, K., Tang, C., Toner, J.J., Covey, D.F., Cistola, D.P. (2002) Proc Natl Acad Sci U S A 99, 1847-52.
43. Nathan, D.F. & Lindquist, S. (1995) Mol Cell Biol 15, 3917-25
44. Dehner, A., Furrer, J., Richter, K., Schuster, I., Buchner, J. & Kessler, H. (2003) Chembiochem 4, 870-7
45. Salek, R.M., Williams, M.A., Prodromou, C., Pearl, L.H. & Ladbury, J.E. (2002) J Biol Mol NMR 23, 327-8
46. Tsai, F.T., Singh, O.M., Skarzynski, T., Wonacott, A.J., Weston, S., Tucker, A., Paempt, R.A., Breeze, A.L., Poyser, J.P., O'Brien, R., Ladbury, J.E. & Wigley, D.B. (1997) *Proteins* **28**, 41-52
47. Huai, Q., Wang, H., Liu, Y., Kim, H.Y., Toft, D. & Ke, H. (2005) *Structure (Camb)* **13**, 579-90
48. Xu, Y. & Lindquist, S. (1993) *Proc Natl Acad Sci U S A* **90**, 7074-8
49. Smith, D.F. (1993) *Mol Endocrinol* **7**, 1418-29
50. Siligardi, G., Hu, B., Panaretou, B., Piper, P.W., Pearl, L.H. & Prodromou, C. (2004) *J Biol Chem* **279**, 51989-98
51. Sullivan, W.P., Owen, B.A. & Toft, D.O. (2002) *J Biol Chem* **277**, 45942-8
52. Johnson, J.L. & Toft, D.O. (1994) *J Biol Chem* **269**, 24989-93
53. Grenert, J.P., Johnson, B.D. & Toft, D.O. (1999) *J Biol Chem* **274**, 17525-33
54. Lotz, G.P., Lin, H., Harst, A. & Obermann, W.M. (2003) *J Biol Chem* **278**, 17228-35
55. Meyer, P., Prodromou, C., Liao, C., Hu, B., Roe, S.M., Vaughan, C.K., Vlasic, I., Panaretou, B., Piper, P.W. & Pearl, L.H. (2004) *Embo J* **23**, 511-9
56. Meyer, P., Prodromou, C., Hu, B., Vaughan, C., Roe, S.M., Panaretou, B., Piper, P.W. & Pearl, L.H. (2003) *Mol Cell* **11**, 647-58
57. Roe, S.M., Ali, M.M., Meyer, P., Vaughan, C.K., Panaretou, B., Piper, P.W., Prodromou, C. & Pearl, L.H. (2004) *Cell* **116**, 87-98
TABLE LEGENDS

TABLE 1. Data collection and refinement statistics for Δ24-N210.

TABLE 2. Estimation of the rotational diffusion behavior of N210 and Δ24-N210. The rotational diffusion behavior has been estimated to distinguish between isotropic, axial symmetric and anisotropic rotational diffusion. The calculations were done as described in Materials and Methods.

TABLE 3. Kinetics of ATP-binding to Hsp90-variants. The ability of the lidless-Hsp90 variants to bind nucleotides and the characteristics of the binding reaction were determined by stopped-flow kinetics at 25 °C. Protein concentrations were 5 μM, and the buffer contained 40 mM HEPES, pH 7.5, 150 mM KCl, 5 mM MgCl2.

TABLE 4. ATPase activity of heterodimers with lidless-Hsp90. The reported activity of the heterodimers was obtained form titration curves as shown in Figure 5. Data analysis was performed based on a fitting algorithm that determines the distribution of species (supplemental data) and estimates the activity of the heterodimeric species. The activity of the homodimeric species was determined independently.
FIGURE LEGENDS

FIGURE 1. Comparison of the crystal structures of Δ24-N210 and wt-N210.
Δ24-N210 (A) and wt-N210 (B) are shown as solid ribbon plots with helices coloured in blue and beta strands coloured in purple. The lid-region (amino acids 99-121), is colored in green, while the first strand (amino acids 1-24), which is deleted in Δ24-N210, is colored in gold. Note the substantial rearrangement of the lid-region of Δ24-N210 (green, A) relative to the corresponding region in wt-N210 (green, B), while the rest of the domain remains largely unchanged. The grey arrowhead marks amino acid 98, the black arrowhead amino acid 121 in Figure 1B. The structure of N210 is based on Prodromou et al. (1997). The PDB access code for this structure is 1AH6.

FIGURE 2. Backbone resonance assignment.
15N HSQC-spectrum of N210 (maroon) and Δ24-N210 (blue). A significant shift of the 15N HSQC peaks was observed for many amino acids after removal of the first 24 amino acids. The assignment of Δ24-N210 is labeled.

FIGURE 3. Dynamics of N210 and Δ24-N210.
A/B, Plot of the squared order parameter versus the sequence of N210 (A) and Δ24-N210 (B). Errors are indicated with error bars. Secondary structure elements are indicated as observed in the crystal structures (arrows: β-strands; bars: α-helices). S² mapped on the crystal structure of N210 (A, left) and Δ24-N210 (B, right). The color ramp is from blue (S²>0.9, restricted motion) to red (S²<0.4, unrestricted motion). Grey areas correspond to residues which showed strong signal overlap in the 15N HSQC or to residues which could not be analyzed or assigned to a motional model. Values for single missing residues were extrapolated using S² values of the next neighbors.
C, The region forming the ATP-lid is conserved in the class of GHKL-ATPases. This can be visualized by the homology of the primary sequence of yeast Hsp90, GyraseB and MutL. Elements of secondary structure (helix, β-strand), of yeast Hsp90 (30) are indicated above the sequence alignment.

FIGURE 4. Structure and stability of lidless-N210.
A, CD-Spectrum of lidless-N210 (dashed line) in comparison to N210 (straight line). The loss in signal between 210 nm to 220 nm reflects the deletion of the two helices forming the ATP-lid. Conditions were as outlined in the Materials and Methods section.
B, Urea-transition of lidless-N210 (○) and N210 (●). Sample preparation and data analysis were performed as described in the Materials and Methods section.
C, Displacement of (Pγ)-MABA-ATP from Hsp90. Complexes of fluorescently labeled nucleotide and different Hsp90 variants were formed and subsequently chased with 100-fold excess of unlabeled nucleotide. The time-dependent decrease in fluorescence represents dissociation of (Pγ)-MABA-ATP from the protein. Analysis with a single exponential equation results in rate constants for dissociation of the nucleotide as shown in table 3. (○) wt-Hsp90, (△) lidless-Hsp90, (Δ) Δ24-Hsp90, (▼) Δ24-lidless-Hsp90.

FIGURE 5. ATPase activity of heterodimers with lidless-Hsp90.
Formation of heterodimers of lidless-Hsp90 with wt-Hsp90. Lidless-Hsp90 has no detectable ATPase-activity. Different concentrations of lidless-Hsp90 were added to wt-Hsp90. The ATPase-activity was determined and the data analysis performed as described in Materials and Methods.
FIGURE 6. Quaternary structure of Hsp90-constructs.
Size-exclusion chromatography of wt-Hsp90 (A), lidless-Hsp90 (B) and Δ24-lidless-Hsp90 (C). Protein concentrations of 35 nM (dotted lines) and 3.5μM (straight lines) were injected. The proteins were detected by intrinsic fluorescence. Conditions were as outlined in the Materials and Methods section.

FIGURE 7. ATPase-properties of Δ24-lidless Hsp90
The activity of heterodimers was measured for the combination of wt-Hsp90 and Δ24-lidless-Hsp90 (○) compared to wt-Hsp90/lidless-Hsp90 (●) and wt-Hsp90/Δ24-Hsp90 (▼). Assay conditions were as outlined in the Materials and Methods section.

FIGURE 8. Model for the proposed conformational changes preceding the N-terminal dimerization reaction.
Depicted are the steps, which lead from an N-terminally monomeric, inhibited state to a dimerized, active state (upper panel). The stable structure of helix 1 (blue) and the lid-region (red) precludes N-terminal dimerization. After this structure is opened (in the presence of ATP; ATP=T, ADP=D, Pi=inorganic phosphate), the dimerization site becomes accessible and the N-terminal strands are exchanged to form the ATPase-active state. This reaction is strongly facilitated if the lid-region is deleted (lower panel), leading to a permanently dimerized state. However, ATP hydrolysis does not occur. It should be noted that efficient hydrolysis requires in addition the association of the N-terminal domain with the middle domain of Hsp90. For simplicity this step is not presented here.
Table 1

Data collection

| Parameter                                           | Value          |
|-----------------------------------------------------|----------------|
| Space group                                         | C2             |
| Cell constants                                      |                |
| A                                                   | 90.50 Å        |
| B                                                   | 52.95 Å        |
| C                                                   | 82.24 Å        |
| β                                                   | 122.05°        |
| Limiting resolution                                 | 1.94 Å         |
| Reflections measured                                | 137 206        |
| $R_{merge}$: overall, outermost shell (1.94-2.04 Å) | 0.055, 0.197   |
| Unique reflections                                  | 22 287         |
| Completeness: overall, outermost shell (1.94-2.04 Å)| 88.3 %, 88.3 % |
| Multiplicity                                        | 6.2            |

Refinement

| Parameter                                           | Value          |
|-----------------------------------------------------|----------------|
| Reflections used for refinement                     | 22 195         |
| Resolution range                                    | 15.0-1.94 Å    |
| Completeness: overall, outermost shell (1.94-2.06 Å)| 88.2 %, 85.4 % |
| R value: overall, outermost shell (1.94-2.06 Å)     | 0.231, 0.247   |
| $R_{free}$: overall, outermost shell (1.94-2.06 Å)  | 0.269, 0.303   |
| Test set size                                       | 4.4 %          |
| RMS standard deviations: Bond length                | 0.011 Å        |
| Bond angles                                         | 1.5°           |
| Average B-value                                     | 22.2 Å²        |
| Ramachandran plot: most favored region              | 85%            |
| favored region                                      | 15%            |
| Non-hydrogen protein atoms                          | 2897           |
| Solvent molecules                                   | 249            |
| Tensor $^e$ | $\tau_c$ (ns)$^a$ | $D_{\text{ratio}}$$^b$ | $\chi^2$$^c$ | $F$$^d$ |
|-------|------------------|-----------------|----------|--------|
|       | N210             | $\Delta$24-N210 | N210     | $\Delta$24-N210 | N210     | $\Delta$24-N210 |
| Isotropic | 10.90 ± 0.01 | 10.01 ± 0.02 | - | - | 4202 | 548 | - | - |
| Axial symmetric$^f$ | 10.81 ± 0.02 | 10.00 ± 0.02 | 1.19 ± 0.01 | 1.13 ± 0.02 | 3406 | 501 | 11.08 | 4.49 |
| Anisotropic$^g$ | 10.80 ± 0.01 | 9.97 ± 0.03 | 1.20 ± 0.01 | 1.13 ± 0.02 | 3329 | 493 | 1.62 | 0.86 |
| Modelfree$^h$ | 10.70 ± 0.02 | 9.89 ± 0.03 | 1.12 ± 0.01 | 1.17 ± 0.02 | - | - | - | - |
| HydroNMR$^i$ | 11.18 | 10.55 | 1.17 | 1.15 | - | - | - | - |

$a$ Rotational correlation time obtained from the relation $\tau_c = 1/(6D_{\text{iso}})$

$b$ Ratio of the components of the diffusion tensor (not for isotropic diffusion)

$c$ summed squared error between experimental and back-calculated data

$d$ F-Test, a high value indicates statistical significance of a complicated diffusional model compared to a simpler one based on Monte-Carlo simulations using the programs r2r1_tm and quadratic_diffusion.

$e$ Anisotropy of the diffusion tensor

$f$ $D_{\text{ratio}} = D_\parallel / D_\perp$

$g$ $D_{\text{ratio}} = 2D_{zz} / (D_{xx} + D_{yy})$

$h$ Optimized Modelfree results

$i$ Results from hydrodynamic calculations using the program Hydronmr
Table 3

| Protein Variant | $k_{on}$ [μM$^{-1}$ s$^{-1}$] | $k_{off}$ [s$^{-1}$] | $K_{D}$, MABA-ATP [μM] | Oligomeric state |
|-----------------|-------------------------------|---------------------|------------------------|-----------------|
| wt-Hsp90        | 0.13 ± 0.05                   | 2.0 ± 0.6           | 15                     | Dimer           |
| wt-N210         | 0.13 ± 0.04                   | 2.3 ± 1.25          | 18                     | Monomer         |
| wt-N530         | 0.14 ± 0.05                   | 1.7 ± 0.4           | 12                     | Monomer         |
| Δ24-Hsp90       | 0.26 ± 0.07                   | 2.9 ± 0.6           | 11                     | Dimer           |
| Δ24-N210        | 0.47 ± 0.13                   | 5.4 ± 1.6           | 11                     | Monomer         |
| Lidless-Hsp90   | 0.12 ± 0.06                   | 1.6 ± 0.5           | 13                     | Dimer           |
| Lidless-N210    | 0.12 ± 0.07                   | 2.3 ± 1.2           | 19                     | Monomer         |
| Δ24-lidless-Hsp90 | 0.55 ± 0.13             | 14.9 ± 1.3          | 27                     | Dimer           |
| Lidless-N530    | 0.18 ± 0.07                   | 1.5 ± 0.3           | 8                      | Monomer         |
Table 4

| Addition of Lidless-Hsp90 | Spec. activity Homodimer [(μM ATP)/(μM Hsp90*time)] | Spec. activity Heterodimer [(μM ATP)/(μM Hsp90*time)] |
|--------------------------|--------------------------------------------------|--------------------------------------------------|
| wt-Hsp90                 | 1.1 ± 0.2                                        | 9.5 ± 2.4                                        |
| Δ8-Hsp90                 | 1.3 ± 0.2                                        | 8.7 ± 2.6                                        |
| Δ16-Hsp90                | 0 ± 0.05                                         | 1.1 ± 0.7                                        |
| Δ24-Hsp90                | 0 ± 0.05                                         | 0 ± 0.05                                         |
| D79N-Hsp90               | 0 ± 0.05                                         | 0 ± 0.05                                         |
| E33A-Hsp90               | 0 ± 0.05                                         | 0 ± 0.05                                         |
Figure 1

(A) 

Δ24-N210

(B) 

N210
Figure 2
Figure 3

(A) and (B) Show structural diagrams of Hsp90 and ATP-lid regions.

(C) Depicts the sequence alignment of Hsp90, MutL, and GyrB with highlighted deletions.

Hsp90: 1-140
MutL: 1-114
GyrB: 67-137
Figure 4

(A) 

(B) 

(C) 

Wavelength (nm) 

MRW (deg^2 dmol^{-1}) 

time (s) 

Urea (M) 

fraction folded 

[4, 6, 8] 

Fluorescence (a.u.) 

[4, 6, 8] 

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Figure 5

![Graph showing specific activity vs. [lidless-Hsp90] (µM)](image-url)

- **Specific activity** (µM\textsubscript{ATP}/(µM\textsubscript{Hsp90}*min))
- **[lidless-Hsp90] (µM)**
Figure 6

(A) Retention Time (min)

(B) Retention Time (min)

(C) Retention Time (min)
Figure 7

[Graph showing specific activity (µM ATP/(µM Hsp90 * min)) against [Hsp90-mutant] (µM).]
Figure 8

Hsp90 wt:

Deletion Mutant:
Supplemental data 1

Estimation of $K_D$ for Δ24-lidless-Hsp90 based on the gel filtration experiment.

Varying concentrations of Δ24-lidless-Hsp90 were injected onto the gel filtration HPLC-system and the shift of the peak was monitored. Data analysis was performed as described previously (3).

![Graph showing dimerization of Δ24Hsp90-lidless](image-url)
Supplemental data 2

Equation for the data analysis of heterodimer assays.

Data analysis of the heterodimer assays was based on a model assuming the equilibrium distribution of homo- and heterodimeric versions, as determined by the two homodimerization constants $K_{\text{Hom}1}$ and $K_{\text{Hom}2}$ and the heterodimerization constant $K_{\text{Het}}$. To calculate the concentration of the monomeric species, the following equation system was solved:

1. $[MM] = \frac{[M] \cdot [M]}{K_{\text{Hom}1}}$
2. $[PP] = \frac{[P] \cdot [P]}{K_{\text{Hom}2}}$
3. $[MP] = \frac{[M] \cdot [P]}{K_{\text{Het}}}$
4. $M_{\text{tot}} = [M] + [MP] + 2 \cdot [MM]$
5. $P_{\text{tot}} = [P] + [MP] + 2 \cdot [PP]$

This resulted in the following equation, describing the concentration of the monomeric species $[P]$:

$$a \cdot [P]^4 - b \cdot [P]^3 + c \cdot [P]^2 + d \cdot [P] + e = 0$$

$$a = \frac{64 \cdot K_{\text{Het}}^2}{K_{\text{Hom}1}^2 \cdot K_{\text{Hom}2}^2} - \frac{16}{K_{\text{Hom}1} \cdot K_{\text{Hom}2}}$$

$$b = \frac{16 \cdot K_{\text{Het}}}{K_{\text{Hom}1} \cdot K_{\text{Hom}2}} - \frac{64 \cdot K_{\text{Het}}^2}{K_{\text{Hom}1}^2 \cdot K_{\text{Hom}2}^2} + \frac{8}{K_{\text{Hom}1}}$$

$$c = -\frac{8 \cdot K_{\text{Het}}}{K_{\text{Hom}1}} + \frac{16 \cdot K_{\text{Het}}^2}{K_{\text{Hom}1}^2} - \frac{64 \cdot K_{\text{Het}}^2}{K_{\text{Hom}1}^2 \cdot K_{\text{Hom}2}^2} \cdot \frac{P_{\text{tot}}}{K_{\text{Hom}1}} + \frac{8 \cdot P_{\text{tot}}}{K_{\text{Hom}1}} - \frac{8 \cdot M_{\text{tot}}}{K_{\text{Hom}1}}$$

$$d = \frac{8 \cdot P_{\text{tot}} \cdot K_{\text{Het}}}{K_{\text{Hom}1}^2} - \frac{32 \cdot P_{\text{tot}} \cdot K_{\text{Het}}^2}{K_{\text{Hom}1}^2}$$

$$e = \frac{16 \cdot P_{\text{tot}}^2 \cdot K_{\text{Het}}^2}{K_{\text{Hom}1}^3}$$
[P] was obtained by numerical methods during the fitting routine and used to derive the values for [M], [MP], [MM] and [PP]. The turnover number was then fitted to the following equation, assuming that the turnover originates only from hydrolysis in the active homodimer MM and the heterodimer MP, as monomeric species are barely populated under the conditions used and PP (lidless-Hsp90) was shown to be inactive in this case. The program SCIENTIST was used for the data analysis as described by Tochtrop et al. (42):

\[
v = \frac{2 \cdot v_1 \cdot [MM] + v_{het} \cdot [MP]}{M_{tot}}
\]

Values for \(v_1\), \(v_2\) (= 0 for lidless-Hsp90), were based on the independently determined ATPase activities of the homodimeric versions and fixed during the data fitting. \(K_{Hom1}\) and \(K_{Hom2}\) were estimated based on the gel filtration experiments and kept invariant as well. \(M_{tot}\) is the invariant concentration of wt-Hsp90 (or \(\Delta8\)-Hsp90 or \(\Delta16\)-Hsp90), which only left \(K_{Het}\) and \(v_{Het}\) as variables during the data analysis. While \(K_{Het}\) is subject to large errors due to its strong dependency on the estimated values \(K_{Hom1}\) and \(K_{Hom2}\), the determination of \(v_{Het}\) is reasonably accurate. Therefore only the turnover number of the heterodimer \(v_{Het}\) is mentioned in the results section.
Intrinsic inhibition of the Hsp90 ATPase activity
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