Hsp90 is a dimeric, ATP-regulated molecular chaperone. Its ATPase cycle involves the N-terminal ATP binding domain (amino acids (aa) 1–272) and, in addition, to some extent the middle domain (aa 273–528) and the C-terminal dimerization domain (aa 529–709). To analyze the contribution of the different domains and the oligomeric state on the progression of the ATPase cycle of yeast Hsp90, we created deletion constructs lacking either the C-terminal or both the C-terminal and the middle domain. To test the effect of dimerization on the ATPase activity of the different constructs, we introduced a Cys residue at the C-terminal ends of the constructs, which allowed covalent dimerization. We show that all monomeric constructs tested exhibit reduced ATPase activity and a decreased affinity for ATP in comparison with wild type Hsp90. The covalently linked dimers lacking only the C-terminal domain hydrolyze ATP as efficiently as the wild type protein. Furthermore, this construct is able to trap the ATP molecule similar to the full-length protein. This demonstrates that in the ATPase cycle, the C-terminal domain can be replaced by a cystine bridge. In contrast, the ATPase activity of the artificially linked N-terminal domains remains very low and bound ATP is not trapped. Taken together, we show that both the dimerization of the N-terminal domains and the association of the N-terminal with the middle domain are important for the efficiency of the ATPase cycle. These reactions are synergistic and require Hsp90 to be in the dimeric state.

Hsp90\(^1\) is an abundant molecular chaperone in the cytosol of eukaryotes and prokaryotes. In contrast to other chaperones, a number of \emph{in vivo} substrates are known for Hsp90 (1, 2). Studies in eukaryotes revealed the specific association of Hsp90 with proteins involved in signal transduction such as tyrosine kinases and steroid hormone receptors (3). Consistent with the importance of the substrate proteins, Hsp90 was found to be an essential protein in yeast (4). The mechanism of Hsp90-mediated client protein activation is not completely understood. In general, Hsp90 is thought to maintain an otherwise unstable conformation of a substrate (1). This reaction seems to involve the ATPase activity of Hsp90, because ATP binding and hydrolysis were found to be essential for the function of Hsp90 (5, 6). Furthermore, geldanamycin and radicicol, which are potent competitive inhibitors of the ATPase (7, 8), prevent cell proliferation (9, 10). The ATP hydrolysis cycle seems to involve the entire Hsp90 molecule (11).

Hsp90 is a dimeric protein. The dimerization site was shown to reside in the very C-terminal domain of Hsp90 (12). For yeast Hsp90, a dissociation constant of 60 nM was determined (13). The C-terminal domain is followed by the middle domain. This domain, for which the three-dimensional structure was reported recently (14), seems to have a number of different functions.

The N-terminal domain of Hsp90 contains a unique nucleotide binding site that differs substantially from that of other chaperones. This site was identified in the crystal structure with either ADP or the Hsp90-specific inhibitor geldanamycin (15, 16). According to this structure, it has been proposed that Hsp90 belongs to the GHKL superfamily of ATP binding proteins that includes the DNA repair protein MutL, DNA gyrase, topoisomerase II, and histidine kinases (17). The nucleotide is bound in an unusual kinked conformation with the adenosine moiety buried and the phosphate groups pointing to the outside of the molecule. The \(\beta\)-phosphate and probably even more the \(\gamma\)-phosphate of ATP are solvent-accessible in the crystal structure. Although the crystal structures of the N-terminal domain of Hsp90 in the presence and absence of nucleotide did not reveal significant structural differences, large conformational changes, as observed for other chaperones (18–20), seem to occur during the ATPase cycle (11, 21, 22). The kinetic analysis of the ATPase cycle revealed that Hsp90 has a weak affinity for ATP (16, 23).

After binding, a conformational change in Hsp90 traps the ATP molecule (11). This trapped ATP molecule is committed to hydrolysis and resistant to exchange for unbound ATP. Because the isolated, monomeric N-terminal domain was not able to trap ATP (11), trapping seems to require additional parts of the protein or the second N-terminal domain. In this context, it had been shown that the N-terminal domains of the full-length protein associate during the ATPase cycle and that this association is required for efficient catalysis (13, 25). N-terminal deletion variants of yeast Hsp90 lacking the first 24 amino acids are severely impaired in their ability to hydrolyze ATP (26). Therefore this region seems to be critical for the N-terminal association of Hsp90. Furthermore, fragments of yeast
Hsp90 lacking C-terminal domains were considerably less active than full-length Hsp90 (11).

To address the contribution of the different domains to the ATPase reaction, we designed constructs comprising the N-terminal domain or the N-terminal domain together with the middle domain. Additionally, we included a cysteine residue at the C terminus of these constructs, which allowed us to produce correctly folded dimers. Hsp90 does naturally contain cysteine residues. The analysis of mono- and dimeric domain constructs of Hsp90 revealed that in the Hsp90 dimer both the N-terminal and the middle domain contribute in a synergistic manner to the progression of the ATPase cycle.

EXPERIMENTAL PROCEDURES

Materials—Geldanamycin was a kind gift of the NCI Experimental Drug Division, National Institutes of Health (Bethesda, MD).

Bacterial Strains—Escherichia coli K12 derivative JM109 (recA1 endA1 gyrA96 thi hsdR17 relA1 supE44 proAB lacIq proAB lacY1 lacZ58 MD15) was used for cloning and propagation, whereas strain M15(pREP4) (Km^R Na^r Sm^r p^f lac ara gal mtl F^ rec^A_1) was used as the expression host for the pQE vectors. All strains were grown in LB or DYT (1.6% Bacto tryptone, 1% yeast extract, 0.5% NaCl, pH 7). Ampicillin was added at a final concentration of 100 μg/ml and kanamycin at 35 μg/ml.

Hsp90 Constructs—The template for amplification of fragments using polymerase chain reactions was the full-length Hsp90 gene of Saccharomyces cerevisiae. We chose the pQE30 vector of the Qiagen system (Qiagen, Hilden, Germany) to clone the wild-type (wt) Hsp90 gene and fragments adding an N-terminal His tag to all proteins expressed. The pQES30 plasmids containing the N- and C-terminal Hsp90 fragments N527Cys (amino acids 1–527-GSA-C-G), 529C (529–709) and N272Cys (1–272-GSA-C-G) were cloned via BamHI and PstI into pQES30. The extension “Cs” denotes the addition of a linker consisting of the amino acids glycine, serine, alanine, a cysteine residue, and glycine as the C-terminal amino acid.

Protein Expression and Purification—M15(pREP4) was grown at 37 °C in dYT-Amp/Kan medium until an_600 reached 0.6–0.8. Expression of His-tagged wt-Hsp90 and fragments was induced by the addition of 20 mM isopropyl-1-thio-β-D-galactopyranoside (final concentration). Cells were lysed using a cell disruption system (Constant Systems, Warwich, UK). All His-tagged proteins were purified on a chelating nickel-nitrotriacetic acid-Sepharose column (Amersham Biosciences) preloaded with 100 mM NiSO_4. Cell lysis and loading of the proteins was performed in loading buffer (100 mM K_HPO_4/K_HPO_4, 300 mM KCI, 5 mM imidazole, pH 8). The column was washed with washing buffer (100 mM K_HPO_4/K_HPO_4, 300 mM KCI, 20 mM imidazole, pH 8) before elution was performed by a step gradient with buffer containing 300 mM imidazole (collection buffer). In a next step, a ResourceQ column (Amersham Biosciences) was used to further purify the proteins. The protein was applied in 40 mM Tris/HCl, 20 mM KCI, pH 8, and eluted with a gradient from 20 to 500 mM KCI in the same buffer. As a final purification step, a Superdex 200 HiLoad (wt-Hsp90, N527Cys) or Superdex 75 HiLoad (529C, N272Cys) (Amersham Biosciences) was employed in 40 mM Hepes/KOH, 300 mM KCI, pH 7.5. Proteins were stored in 40 mM Hepes/KOH, 20 mM KCI, 5% glycerol, pH 7.5, at concentrations of 3–10 mg/ml at –80 °C.

Formation of Disulfide Bonds—After ion exchange chromatography, disulfide bonds between the monomeric fragments were formed under oxidizing conditions by aeration of the buffer. Monomers and dimers were subsequently separated using the size exclusion chromatography columns Superdex 200 HiLoad and Superdex 75 HiLoad, respectively.

Radioactive ATPase Assay—The radioactive ATPase assays were performed according to Kornberg et al. (27). Hsp90 and the N-terminal fragments were incubated in 40 mM Hepes/KOH, 150 mM KCI, 5 mM MgCl_2, pH 7.5, at 37 °C. For the N-terminal fragments and wt-Hsp90, a concentration of 0.5 μM was used in each assay. Hsp90 and fragment ATPase activities are expressed as mCi (milli-curie) of [γ-^32P]ATP (Hartmann Analytic, Braunschweig, Germany). The protein to ATP ratio was kept constant at 1.0. ATP hydrolysis was stopped at individual time points by the addition of 80 μM EDTA, and the samples were spotted on polyethyleneimine-cellulose plates (Merck, Darmstadt, Germany). Thin-layer chromatography was performed in 0.5 M LiCl and 2 M formic acid. Plates were dried under red light, and the ATP to ADP ratio was quantified using a Typhoon 9200 PhosphorImager (Amersham Biosciences). Hydrolysis rates were corrected for uncatalysed, spontaneous ATP hydrolysis. Quenching the ATPase reaction with trichloroacetic acid gave results that were identical with those obtained by quenching with EDTA. Inhibition of ATPase activity was achieved by the addition of 5 mM geldanamycin dissolved in MeSO. In control experiments, 1% MeSO alone did not affect the ATPase activities.

Nucleotide Binding—Stopped-flow measurements were performed with a Hitachi SF-61 DX2 instrument in 40 mM Hepes/KOH, pH 7.5, 150 mM KCI, 5 mM MgCl_2. The excitation slit was 0.5 nm, the excitation wavelength was 334 nm, and emission was detected through a cut-off filter above 418 nm. The temperature was set to 25 °C unless indicated otherwise.

Typically, each experiment was performed 6–10 times, and the resulting time traces were averaged with the HiTech Scientific software. Displacement experiments were performed with an excess of displacing ligand to ensure irreversible displacement; otherwise the observed rate constant for displacement would constitute a mixture of dissociation and rebinding kinetics (28). In the case of experiments measuring the association of fluorescent ligands, either the concentration of enzyme or that of the ligand was at least 3-fold in excess to ensure proper pseudo-first-order binding kinetics (29).

The primary data were analyzed using HiTech Scientific software with the following single exponential equations for the displacement reaction (Equation 1) and for the binding of (Py)-MABA-ATP (Equation 2).

\[
F = F_0 - A(1 - \exp(-kt)) \quad (\text{Eq. 1})
\]

\[
F = F_0 + A(1 - \exp(-kt)) \quad (\text{Eq. 2})
\]

where F is signal, A is amplitude, F_0 is offset, k is observed rate constant, and t is time. The secondary data of binding experiments (observed rate constants as obtained from a single exponential fit of each time trace versus concentration of ligand in excess) were analyzed with a linear equation. This follows from the fact that under pseudo-first-order conditions (one ligand in excess) and for a simple one-step binding mechanism (k_{obs} = k_{on} \times [L]_{excess} + k_{off}), a plot of k_{obs} versus [L]_{excess} gives k_{on} as the intercept and k_{off} as the slope, with [L]_{excess} being the concentration of ligand in excess.

RESULTS

Addition of a Cysteine Residue at the C Terminus of N-terminal Hsp90 Fragments Allows Artificial Dimerization—Earlier studies had shown that C-terminal deletion mutants of Hsp90, which contain the N-terminal ATP-binding site, exhibited considerably lower ATPase activity than the wt protein (11, 13, 30, 31).

We were interested in analyzing how the C-terminal domain (amino acids 529–709) influences the ATP hydrolysis of Hsp90. Because the C-terminal domain mediates dimerization of Hsp90, its function could either be restricted to dimerization, or additionally, it could be directly involved in stimulating the ATPase activity. To differentiate between the two possibilities, we decided to create yeast Hsp90 variants that lack the C-terminal dimerization domain and contain an additional cysteine residue at the C-terminal end. Hsp90 does not naturally contain cysteine. The artificially introduced cysteine allows the covalent linking of the respective Hsp90 monomers and thus the production of artificial dimers. One fragment we chose to use contains amino acids 1–527 (N527Cys) (Fig. 1). A shorter fragment comprising the N-terminal ATP-binding site, exhibited considerably lower ATPase activity than the wt protein (11, 13, 30, 31).

We investigated how the C-terminal domain (amino acids 529–709) influences the ATP hydrolysis of Hsp90. Because the C-terminal domain mediates dimerization of Hsp90, its function could either be restricted to dimerization, or additionally, it could be directly involved in stimulating the ATPase activity. To differentiate between the two possibilities, we decided to create yeast Hsp90 variants that lack the C-terminal dimerization domain and contain an additional cysteine residue at the C-terminal end. Hsp90 does not naturally contain cysteine. The artificially introduced cysteine allows the covalent linking of the respective Hsp90 monomers and thus the production of artificial dimers. One fragment we chose to use contains amino acids 1–527 (N527Cys) (Fig. 1). A shorter fragment comprising the N-terminal ATP binding domain and the charged linker region was also constructed (N272Cys) (27). As a complementary C-terminal fragment we used 529C, which contains amino acids 529–709 (Fig. 1). This protein represents the C-terminal dimerization domain, which is able to form heterodimers with Hsp90 (13, 32).

The structure and stability of the Hsp90 fragments were analyzed by CD spectroscopy and urea-induced unfolding transitions. All fragments are structured and exhibit cooperative unfolding transitions with midpoints of unfolding between 3.5 and 4.5 M urea (data not shown).

The dimeric species of N527Cys and N272Cys migrated at the expected positions in the gel (Fig. 2). Samples that had been

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incubated with a reducing agent before separation migrated at the position of the respective monomer (Fig. 2). SDS-PAGE confirmed that the dimers were covalently linked. Taken together, these experiments show that it is possible to produce dimeric Hsp90 fragments that are linked via a C-terminal disulfide bond.

Artificial Dimerization of N-terminal Fragments Leads to Increased ATP Hydrolysis—Dimerization of wt-Hsp90 is a dynamic process allowing the formation of heterodimers (13, 32). If a monomer-dimer transition mediated via the C-terminal dimerization domain was an important step in the ATPase cycle of yeast Hsp90, covalently linked dimers would be compromised in the ATPase activity. Similarly, if the C-terminal domain was required for specific interaction in addition to providing a dimerization site, a negative influence on the ATPase reaction would be expected.

Analyzing monomeric and dimeric Hsp90 fragments, we found that the ATPase of the N527Cys monomer ($k_{\text{cat}}$) was $0.15 \pm 0.05 \text{ min}^{-1}$ (Table I, Fig. 3A), whereas that of the N527Cys dimer was $1.1 \pm 0.2 \text{ min}^{-1}$ (Table I, Fig. 3A). Thus, the activity of the dimer is seven times higher than the activity of the monomer and even 100% higher than the activity of wt-Hsp90. In a control experiment, we added reducing agent to the dimeric N527Cys fragment. This addition resulted in the reduction of the disulfide bond and the subsequent monomerization of the N527Cys fragment. Concomitantly, the ATPase activity decreased to the levels of the monomer (Fig. 3B). These results show that artificial dimerization of N527Cys by a C-terminal disulfide bond is sufficient for wt ATPase activity.

Next, we wanted to determine whether the middle domain was also obsolete in the presence of a covalent linkage. To test this, we used artificially dimerized N272Cys fragments, consisting only of the ATP binding domain and the charged linker. In agreement with previous results, the monomeric species of N272Cys exhibited an ATPase activity of about $0.007 \pm 0.003 \text{ min}^{-1}$, which is less than 1% of wt activity (11, 13, 30). The covalent N272Cys dimer showed an ATPase activity of $0.015 \pm 0.005 \text{ min}^{-1}$. This means that dimerization of the N-terminal ATP binding domain increased the ATPase activity by a factor of approximately 2. However, it should be noted that this value represents only 2% of the activity of wt-Hsp90 or of the larger dimeric fragment N527Cys.

Because of the calculated dissociation constant ($K_D$) of 60 nM for dimeric yeast Hsp90 (13), the direct determination of the activity of monomeric wt-Hsp90 is practically impossible. To solve this problem, we used the C-terminal fragment 529C, which comprises the dimerization domain. The addition of an excess of the C-terminal fragment 529C to wt-Hsp90 results in the formation of wt-Hsp90·529C heterodimers and 529C homodimers. As the C-terminal fragments show no ATPase activity, only the wt-Hsp90·529C heterodimer contributes to the ATPase activity measured. Thus, the ATPase activity of the heterodimer corresponds to the activity of the wt-Hsp90 monomer. When increasing amounts of 529C (1–100 μM) were added to wt-Hsp90 (2 μl), a concentration-dependent decrease in the ATPase activity was observed (Fig. 3C). The rate constant ($k_{\text{cat}}$) of ATP hydrolysis for the wt-Hsp90·529C heterodimer was $0.17 \pm 0.02 \text{ min}^{-1}$ compared with $1.0 \pm 0.2 \text{ min}^{-1}$ for the wt-Hsp90 homodimer. This means that dimeric wt-Hsp90 hydrolyzes ATP by a factor of 5 more efficiently than the respective monomeric species. Furthermore, the addition of 529C to N527Cys-D and N527Cys-M did not appear to have any effect on their ATPase activities (Fig. 3C).

**TABLE I**

| Protein                  | $k_{\text{cat}}$ ($\text{min}^{-1}$) | $K_m$ (μM) |
|--------------------------|-------------------------------------|------------|
| wt-Hsp90                 | 1.0 ± 0.2                           | 350 ± 40   |
| wt-Hsp90·529C            | 0.17 ± 0.02                         | ND         |
| Hsp90·N527Cys dimer      | 0.15 ± 0.05                         | 520 ± 40   |
| Hsp90·N272Cys dimer      | 0.015 ± 0.005                       | 760 ± 100  |
| Hsp90·N272Cys-M          | 0.007 ± 0.003                       | 1000 ± 150 |

ATP Binding Properties of Hsp90 Fragments—To gain more insight into the underlying mechanistic differences of the ATPase of Hsp90 fragments, we determined their $K_m$ values for ATP (Fig. 4). The rate constants ($k_{\text{cat}}$) measured exhibited a hyperbolic dependence on the ATP concentrations (50 μM to 4 mM ATP). A plot of $k_{\text{cat}}$ versus [ATP] (Lineweaver-Burk plot, not shown) allowed us to determine the apparent $K_m$ values for the different constructs.

The limiting rate constants ($k_{\text{cat}}$) for ATP hydrolysis of wt-Hsp90, N527Cys-D, N527Cys-M, N272Cys-D, and N272Cys-M were determined to be $1.0 ± 0.2 \text{ min}^{-1}$, $1.1 ± 0.2 \text{ min}^{-1}$, $0.15 ± 0.05 \text{ min}^{-1}$, $0.015 ± 0.005 \text{ min}^{-1}$, and $0.007 ± 0.003 \text{ min}^{-1}$, respectively, at saturating ATP concentrations (see Table I).

As shown in Table I, the $K_m$ values of wt-Hsp90 and N527Cys-D do not differ significantly, whereas the $K_m$ of N272Cys-M is lower by a factor of 1.5. The $K_m$ values of N272Cys-M and N272Cys-D differ slightly and are about three times lower than with wt-Hsp90. This leads to the assumption...
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Kinetics of ATP Binding to Hsp90 Fragments—To further analyze the nucleotide binding properties of the monomeric and dimeric fragments lacking the C-terminal domain in comparison to wt-Hsp90, we performed stopped-flow experiments with (Pγ)-MABA-ATP, a fluorescently labeled ATP analogue. (Pγ)-MABA-ATP emits fluorescence at 418 nm when excited at 334 nm, and this fluorescence increases upon binding to Hsp90 (11). This allowed us to determine association and dissociation rate constants for the interaction of Hsp90 fragments with ATP.

First, we determined the release rate for the nucleotide by direct displacement of (Pγ)-MABA-ATP from the preformed complexes with an excess of unlabeled nucleotide. Complexes of (Pγ)-MABA-ATP and each wt-Hsp90, N527Cys-M, and N527Cys-D were incubated for 30 min to allow complex formation. Subsequently, this solution was rapidly mixed with an excess of unlabeled ATP to ensure complete displacement. Thus, the observed rate constant will be equal to the rate constant for dissociation (k_{off}) of (Pγ)-MABA-ATP.

The reactions followed a single exponential function with observed rate constants of 2.57 ± 0.2 s\(^{-1}\), 2.90 ± 0.2 s\(^{-1}\), and 2.71 ± 0.2 s\(^{-1}\) for wt-Hsp90, N527Cys-D, and N527Cys-M, respectively (Fig. 5, A–C; Table II). Each of these experiments was performed at different protein concentrations, and the observed rate constants were plotted versus protein concentration (data not shown). The binding of (Pγ)-MABA-ATP to Hsp90 and fragments can be described by a simple one-step reversible reaction. The slopes of the least square fitted solid lines reveal the rate constants for association (k_{on}) of (Pγ)-MABA-ATP to wt-Hsp90 and the N-terminal fragments (28).

The calculated K_m values of wt-Hsp90 and the N-terminal fragments, ATPase assays were performed in the presence of ATP concentrations ranging from 50 μM to 4 mM. Each rate constant (k_{on}) represents the mean of three independent experiments. A, wt-Hsp90; B, N527Cys-D; C, N527Cys-M. B, C, N272Cys-D; D, N272Cys-M.

![Fig. 3. ATPase activities of mono- and dimeric N-terminal Hsp90 fragments. A, ATPase activities of wt-Hsp90, N272Cys, and N527Cys fragments. The ATPase activities were determined using a radioactive ATPase assay (see “Experimental Procedures”). □, N272Cys-D (5 μM); ○, wt-Hsp90 (5 μM); ▲, N527Cys-M (5 μM); □, N272Cys-D (5 μM). B, reduction of the N527Cys-D results in decreased ATPase activity. The radioactive ATPase assay described under “Experimental Procedures” was used. Dithiothreitol (DTT) was added as the reducing agent to N527Cys dimers at the time point indicated by the arrow. In comparison, the activity of the N527Cys-M is shown. ●, N527Cys dimer (5 μM); ○, N527Cys monomer (5 μM). C, ATPase activity of full-length Hsp90 monomers. The addition of the C-terminal fragment 529C to wt-Hsp90 leads to the formation of wt-Hsp90-529C heterodimers. The concentration of 529C was varied from 1 to 100 μM at constant concentrations of wt-Hsp90 (2 μM, ●), N527Cys-D (2 μM, ▲), and N527Cys-M (2 μM, □).](image)

![Fig. 4. K_m values of Hsp90 variants for ATP. To determine the K_m values of wt-Hsp90 and the N-terminal fragments, ATPase assays were performed in the presence of ATP concentrations ranging from 50 μM to 4 mM. Each rate constant (k_{on}) represents the mean of three independent experiments. A, wt-Hsp90; B, N527Cys-D; C, N527Cys-M. B, C, N272Cys-D; D, N272Cys-M.](image)
dissociation and the rate constant for association ($k_{on}$) (Table II). The reduced binding affinity of N527Cys-M for ATP is in agreement with the slower binding kinetics between (Pγ-MABA-ATP) and N527Cys-M in comparison with wt-Hsp90 and N527Cys-D.

Trapping of ATP by wt-Hsp90 and N-terminal Fragments—Next, we were interested in the ability of the different Hsp90 constructs to trap the bound nucleotide. We had shown previously that wt-Hsp90 protects ATP against exchange during the ATPase cycle, in contrast to the monomeric N-terminal domain (11).

To test the commitment to hydrolysis of ATP bound to N-terminal fragments of Hsp90, we performed single-turnover experiments, in which, at certain time points after complex formation between labeled ATP and Hsp90 fragments, an excess of unlabeled ATP was added. As a consequence, uncommitted ATP will be chased off the Hsp90 fragments, whereas the committed, conformationally trapped ATP is not affected. For wt-Hsp90, N527Cys-D, and N527Cys-M, plots with 10 μM protein and 3 μM (Pγ-MABA-ATP) are shown.

**Table II**

| Protein                  | $k_{on}$ | $k_{off}$ | $K_D$  |
|--------------------------|----------|-----------|--------|
| wt-Hsp90                 | 0.16 ± 0.02 | 2.572 ± 0.2 | 16 ± 0.2 |
| Hsp90-N527Cys dimer      | 0.21 ± 0.02 | 2.889 ± 0.2 | 13.8 ± 0.2 |
| Hsp90-N527Cys monomer    | 0.07 ± 0.02 | 2.708 ± 0.2 | 38.7 ± 0.2 |

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![Figure 5](image)

**Fig. 5.** Nucleotide binding properties of the Hsp90 variants wt-Hsp90, N527Cys-M, and N527Cys-D. A–C, the dissociation rate constant ($k_{off}$) of the fluorescent ATP analogue (Pγ-MABA-ATP) was determined at 25 °C directly by displacement of (Pγ-MABA-ATP) with ATP from wt-Hsp90 and N-terminal fragments. (Pγ-MABA-ATP) and Hsp90 variants were incubated for 30 min to allow complex formation. Subsequently, (Pγ-MABA-ATP) was displaced by an excess of ATP. A, wtHsp90; B, N527Cys-D; C, N527Cys-M. D–F, the binding kinetics ($k_{on}$) of (Pγ-MABA-ATP) and Hsp90 variants were measured at different protein concentrations after rapid mixing. For wt-Hsp90, N527Cys-D, and N527Cys-M, plots with 10 μM protein and 3 μM (Pγ-MABA-ATP) are shown.
As expected, no trapping was observed for N272Cys-D and N272Cys-M. In this case, the \( k_{\text{obs}} \) values were 0.02 \pm 0.005 min\(^{-1}\) and 0.01 \pm 0.005 min\(^{-1}\) (Fig. 6, D and E).

**DISCUSSION**

The ATPase activity of Hsp90 seems to involve regions from the entire molecule. ATP is bound by the N-terminal domain. Subsequently, these domains have to dimerize to achieve efficient catalysis (13, 25), and they may interact with regions in the middle or C-terminal domain as shown by the trapping of the bound nucleotide (11). The relative importance of the individual contributions was, however, unclear. The analysis of the partial reactions is hampered by the contribution of the C-terminal dimerization to the overall reaction. To be able to differentiate between specific domain interactions during the catalytic cycle and the general requirement for a dimeric molecule, we introduced a cysteine residue at the C-terminal end of constructs lacking the C-terminal domain.

In the first set of experiments, we show that decreasing the length of N-terminal yeast Hsp90 fragments leads to a reduction in the ATPase activity. The first decrease (by a factor of 7) in the ATPase activity is observed with fragments lacking the C-terminal dimerization domain. The second drastic decrease (by a factor of about 100 compared with wt-Hsp90) is observed for fragments lacking the middle domain. It is well established that the isolated N-terminal domain exhibits a very low ATPase activity, which corresponds to about only 1% of the activity of the full-length protein (11, 25, 30, 31). If the dimerization of the N-terminal domains was the decisive factor for efficient ATP hydrolysis, a covalent linkage of two N-terminal domains (N272Cys-D) should be sufficient to re-establish full ATPase activity. Our results using a cystine-bridged N-terminal domain (N272Cys-D) show that its intrinsic ATPase can be doubled. However, this activity still amounts only to 2% of the activity of the wild type protein (Table I). Furthermore, there was no detectable trapping of the ATP in the artificially linked N-terminal domains. This result suggested that additional interactions with the middle or C-terminal domain may provide important contributions to the hydrolysis cycle. Although there

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**Fig. 6. Trapping of ATP by wt-Hsp90 and N-terminal fragments.** wt-Hsp90, N527Cys-D, N527Cys-M, N272Cys-D, and N272Cys-M were incubated under single turnover conditions with \([\alpha-\text{32P}]\text{ATP}\). After a certain incubation time, the samples were split, and an excess of unlabeled ATP was added to one-half of the sample (chase), while the other half was supplemented with the same volume of buffer as a control (pulse). A, wt-Hsp90 (1 mM) with 0.8 mM ATP; B, N527Cys-D (1 mM) with 0.8 mM ATP; C, N527Cys-M (1 mM) with 0.8 mM ATP; D, N272Cys-D (3 mM) with 2.4 mM ATP; E, N272Cys-M (3 mM) with 2.4 mM ATP.
is a significant increase in ATPase activity upon dimerization of N272Cys, the ATPase activity of the covalently linked dimer is way below wt-Hsp90 activity. The results of single-turnover and binding constant measurements indicate that there is a region in the middle domain that is responsible for closing the binding pocket, which is essential for full ATPase activity. This is in agreement with the drastic decrease of the ATPase activity observed between N527Cys-M and the N272Cys-M. A construct containing the N-terminal and the middle domain but lacking the C-terminal domain is monomeric (N527Cys-M). This construct exhibits about 15% of the ATPase activity of the full-length protein and it is able to trap ATP. These results strongly suggest that the increase in ATPase activity is due to an interaction of the N-terminal with the middle domain during the ATPase cycle. The oxidized (dimerized) version of this construct (N527Cys-D) exhibited full ATPase activity. This result has several important consequences. First, it shows that the C-terminal domain is not required for the ATPase cycle; its functional contribution can be substituted by a covalent bond. Second, the N-terminal dimerization and the interaction with the middle domain are synergistic. If one could function independent of the other, the sum of the ATPase activities of the dimeric N-terminal domain and that of the monomeric fragment containing the N-terminal and the middle domain should be equal to the activity of the wt protein. The fact that fragments including the N-terminal and middle domain, but not fragments including just the N-terminal and linker domain, are able to exhibit full ATPase activity upon dimerization gives rise to the assumption that parts of the middle domain in Hsp90 are essential for full ATPase activity. For the homologous proteins GyrB, MutL, and CheA, it was shown that the \( \gamma \)-phosphate group of ATP is complexed by a lysine residue from the accompanying domain, leading to a structural reorganization of the domains and the burial of the nucleotide inside the protein (33, 34). It has been suggested previously that the same may be true for Hsp90 (11). The recently solved structure of the middle domain of Hsp90 suggests that a possible candidate for an acceptor of the \( \gamma \)-phosphate group of ATP could be Lys-387, even if it is not well positioned to act as the equivalent catalytic lysines in MutL and GyrB (14). The structure of the middle segment of Hsp90 further supports the view of the evolutionary relationship between Hsp90 and MutL or GyrB. Most of the residues involved in the binding and hydrolysis of the nucleotide are conserved in these proteins. The fact that the \( K_m \) value of N272Cys monomer (\( K_m = 1000 \mu M \)) is nearly 2 times higher than that of N527Cys monomer (\( K_m = 520 \mu M \)) further gives rise to the assumption that regions from the middle domain of Hsp90 take part in ATP binding, as proposed previously (11, 14). Trapping or commitment to hydrolysis usually results from conformational changes in a protein that lock the nucleotide in the nucleotide-binding pocket, where it is hydrolyzed before it can be released. Commitment has already been reported for the chaperone GroEL (18). In the case of Hsp90, trapping occurs only in Hsp90 fragments containing the middle domain. Therefore we conclude that the middle domain of Hsp90 is important for the trapping of the ATP molecule. Our results from the ATP binding studies show that covalently linked N527Cys-D and wt-Hsp90 bind ATP with comparable affinity (\( K_m = 328 \mu M \) and \( K_m = 350 \mu M \), respectively). No significant change in the dissociation constant for (P\(^\gamma\))-MABA-ATP (\( K_D = 13.8 \) and \( 16 \mu M \), respectively) was observed. This indicates that the access of (P\(^\gamma\))-MABA-ATP to the ATP binding pocket of N527Cys-D and wt-Hsp90 is not changed. In the monomer (N527Cys-M), the \( K_D \) and the \( K_m \) values differ from the wt protein by a factor of 2, which indicates that dimerization is important for efficient ATP binding.

For chicken Hsp90\( \alpha \) it had been shown that a monomer, C-terminal truncation construct (amino acids 1–573) leads to a 100-fold increase in the ATPase activity (35). In this case, the C-terminal part of the protein seems to exhibit a negative regulatory effect on the ATPase activity. However, for yeast Hsp90, the results from our trans-complementation studies using the C-terminal fragment 529C show no influence on the ATPase activity of the N527Cys monomer or dimer. In addition, the ATPase activity of the N527Cys monomer equals that of the wt-Hsp90 529C heterodimer, making even a cis-regulatory effect for yeast Hsp90 unlikely. It is therefore reasonable to assume that the ATPase activity of yeast Hsp90 is controlled by mechanisms other than Hsp90 from higher eukaryotes. In this context, also the regulatory function of Sti1/Hsp on the ATPase activity of yeast or human Hsp90 have clearly been shown to be different (24, 36).

Taken together, our analysis allows further dissection of the ATPase cycle of Hsp90. The progression of the ATPase cycle depends critically on the coupling of conformational changes with ATP hydrolysis. Specifically, the synergistic dimerization of the N-terminal domains in the Hsp90 dimer and association with the middle domains seems to determine the enzymatic activity of Hsp90.

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*Individual Domains Contribute to ATPase Mechanism of Hsp90*