The ATPase Cycle of the Mitochondrial Hsp90 Analog Trap1

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Hsp90 is an ATP-dependent molecular chaperone whose mechanism is not yet understood in detail. Here, we present the first ATPase cycle for the mitochondrial member of the Hsp90 family called Trap1 (tumor necrosis factor receptor-associated protein 1). Using biochemical, thermodynamic, and rapid kinetic methods we dissected the kinetics of the nucleotide-regulated rearrangements between the open and the closed conformations. Surprisingly, upon ATP binding, Trap1 shifts predominantly to the closed conformation (70%), but, unlike cytosolic Hsp90 from yeast, this process is rather slow at 0.076 s⁻¹. Because reopening (0.034 s⁻¹) is about ten times faster than hydrolysis (kₜ₉ = 0.0039 s⁻¹), which is the rate-limiting step, Trap1 is not able to commit ATP to hydrolysis. The proposed ATPase cycle was further scrutinized by a global fitting procedure that utilizes all relevant experimental data simultaneously. This analysis corroborates our model of a two-step binding mechanism of ATP followed by irreversible ATP hydrolysis and a one-step product (ADP) release.

90-kDa heat shock protein (Hsp90) is a molecular chaperone highly conserved from bacteria to mammals. The dependence on Hsp90 apparently reflects the relative complexity of the organism. For example, bacteria lacking HtpG (the prokaryotic homolog of mammalian Hsp90) appear to be fully viable (1), whereas disruption of Hsp90 in eukaryotic organisms, including Saccharomyces cerevisiae, Caenorhabditis elegans, and Drosophila, is lethal (2–5). The family of Hsp90-related molecular chaperones mediates protein folding, assembly, and stability of a variety of substrate proteins in vivo. In vitro, Hsp90 suppresses the aggregation of non-native proteins (6) and can also promote the refolding of substrates in cooperation with the Hsp70 system (7–10).

In eukaryotic organisms, several members of the Hsp90 family can be found in different compartments such as the cytosol, endoplasmic reticulum, mitochondria and in chloroplasts (11). The organellar versions differ from their cytosolic counterparts in several aspects. The most striking is that they seem to lack specific co-chaperones. While Grp94, the Hsp90 homolog of the endoplasmic reticulum has evolved from its cytosolic counterpart, Trap1, the mitochondrial version, is a descendant of prokaryotic Hsp90 (11). Whether they share a general mechanism of ATP hydrolysis is not known. In this study, we focused on the biochemical and kinetic analysis of the Hsp90 homolog Trap1, which is localized in mitochondria (12–14). Originally, Trap1 was identified as a tumor necrosis factor receptor-associated protein (15). Trap1 is 31% identical to human Hsp90, 34% to Escherichia coli HtpG, 33% to yeast Hsp90, and 31% to Grp94. For the N-terminal ATP-binding domain alone, the identities are even higher. There are a few identified Trap1 substrates such as the retinoblastoma protein (16), the tumor suppressor EXT proteins (17) and Myc, which influences cell proliferation (18). Recently, an interaction partner of Trap1, which is located inside mitochondria, was reported (19). Cyclophilin D, which is responsible for mitochondrial cell death, binds to Trap1 so that its function upon stress is prevented. This protective pathway, which is mainly detected in tumor cells, can be disrupted by adding the known Hsp90 inhibitors geldanamycin or 17-(allylamino)-17-demethoxygeldanamycin.

It has been established for members of the Hsp90 family that ATP binding and hydrolysis are essential for their in vivo function (20–22). ATP binds in an unusual kinked conformation with the γ-phosphate group pointing to the surface of the N-terminal domain (23–26). This binding mode is only found in a few other protein families such as DNA gyrase and MutL. Together with Hsp90, they are grouped together in the GHKL family (27).

An increasing number of clients has been reported for Hsp90 chaperones from in vivo studies, such as proteins involved in cell cycle regulation, steroid hormone response, and signal transduction (29–34). Because of its involvement in cell cycle regulation, Hsp90 is currently evaluated as a target for anticancer drugs. At the moment, mainly natural compounds such as radicicol (RA) and geldanamycin and their derivatives are tested in this context (24, 35–38). These compounds are inhibitors that bind to the ATP binding pocket in the N-terminal domain of Hsp90 and thus compete with nucleotides for the binding site. Binding of the inhibitors to Hsp90 leads to the
release of bound client proteins and thus to loss of protection/folding assistance (39). The specificity of these drugs for Hsp90 is based on the peculiar structure of nucleotide bound to Hsp90 (23, 36).

The functional role of intermediates of the Hsp90 ATPase cycle is not well understood yet. The kinetics of the ATPase cycle of yeast Hsp90 (40), human Hsp90 (41), and Grp94 (42) had already been investigated in some detail using biochemical approaches. Yeast Hsp90 was observed by biochemical and crystallographic studies to undergo a conformational change upon ATP binding resulting in the so-called closed conformation in which the N-terminal domains are transiently associated (43, 44). However, the rate constants of this step were not accessible (40).

In contrast, even after ATP binding Grp94 stays predominately in the open conformation and thus does not allow to gather direct information about this key step of the cycle (42). Only recently, full-length structures of yeast Hsp90 and HtpG were reported. Yeast Hsp90 in complex with the co-chaperone p23 and AMP-PCP (44) indicated an N-terminally closed conformation as well as HtpG bound to AMP-PCP or ADP (26). HtpG in the apo-form but also Grp94 in the presence of nucleotide revealed an open conformation (26, 48). The important function of the first amino acids of the N-terminal domains in establishing the N-terminal dimerization was analyzed using deletions or point mutations (43). As evidenced by this structural information, nucleotide binding leads to large scale conformational changes throughout the entire Hsp90 molecule. These nucleotide-dependent changes of Hsp90 seem to regulate the highly complex and dynamic interactions of Hsp90 with client proteins and co-chaperones. The crystal structures only describe snap shots but cannot provide information about the kinetics by which functional relevant states interchange nor their balance in solution.

In this report, we investigated the ATPase cycle of Trap1 and determined all relevant equilibria as well as the dynamic properties of the individual steps of the ATPase cycle. We scrutinized the minimal kinetic model with global fit analysis and could not detect the necessity for additional steps prior or following hydrolysis nor could an even reduced model still explain all data. An extension of experimental conditions beyond those reported. Yeast Hsp90 was transformed with pET-Trap1. Cells were grown in LB medium at 37 °C, and protein synthesis was induced with isopropyl-β-D-thiogalactopyranoside (final concentration, 2 mM) at an optical density of 0.6. After induction, cells were grown at 37 °C for 3 h and harvested by centrifugation. The bacterial pellet was resuspended in lysis buffer (20 mM Hepes/KOH, pH 7.5, 0.1 mM EDTA, and 2 mM dithiothreitol) and sonicated after addition of complete protease inhibiting mixture tablets for 20 min on ice. Finally, insoluble and soluble material was separated by centrifugation (186,000 × g, 40 min, 4 °C, 45Ti rotor, Beckman, Krefeld, Germany). The protein was purified at 4 °C by ion exchange chromatography on an EMD-DEAE column (Merk) with a gradient from 0 to 1 M KCl in lysis buffer. After dialysis at 4 °C against 10 mM KP, pH 7.5, 50 mM KCl, and 2 mM dithiothreitol, the pooled fractions were applied to a hydroxyapatite column (Merk) at 25 °C and eluted with a gradient from 0.01 to 0.5 mM potassium phosphate buffer, pH 7.5. As a further purification step, size exclusion chromatography was performed using a Superdex 200 gel filtration column (GE Healthcare, München, Germany) at 4 °C with 20 mM Hepes/KOH, pH 7.5, 400 mM KCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol. After analysis by SDS-PAGE, the gels were Coomassie-stained. The peak fractions containing protein with a molecular mass of 73 kDa were pooled and stored at a concentration of 12 mg/ml in the buffer of the gel filtration at −80 °C. The identity of Trap1 was determined by Western blotting using a polyclonal Trap1 antibody provided by D. Toft (Mayo Clinic) and mass spectrometry. The concentration of Trap1 was determined according to Ehresman (46).

**EXPERIMENTAL PROCEDURES**

**Chemicals**

All chemicals (including nucleotides) were purchased from Sigma, Merck (Darmstadt, Germany), Gerbu Biotechnik (Gaiberg, Germany), Roche Applied Science, or Pharma Waldhof Mannheim (Mannheim, Germany) at the highest quality available. RA was obtained from AG Scientific (San Diego, CA). (Py)-MABA-ATP was prepared as described previously (45).

**Construction of the Plasmid pET-Trap1**

The cDNA sequence of human Trap1, lacking the first 177 bp (Toft, Mayo Clinic, Rochester, MN), was cloned as a restriction fragment into a pET9a vector (Novagen, Madison, WI) using NdeI and BamH1 to express the protein in the cytoplasm of *E. coli*. The identity of the construct was confirmed by automated DNA sequencing. Its calculated molecular mass was 73,547 Da.

**Purification of Trap1**

*E. coli* BL21(DE) Codon Plus cells (Stratagene, La Jolla, CA) were transformed with pET-Trap1. Cells were grown in LB medium at 37 °C, and protein synthesis was induced with isopropyl-β-D-thiogalactopyranoside (final concentration, 2 mM) at an optical density of 0.6. After induction, cells were grown at 37 °C for 3 h and harvested by centrifugation. The bacterial pellet was resuspended in lysis buffer (20 mM Hepes/KOH, pH 7.5, 0.1 mM EDTA, and 2 mM dithiothreitol) and sonicated after addition of complete protease inhibiting mixture tablets for 20 min on ice. Finally, insoluble and soluble material was separated by centrifugation (186,000 × g, 40 min, 4 °C, 45Ti rotor, Beckman, Krefeld, Germany). The protein was purified at 4 °C by ion exchange chromatography on an EMD-DEAE column (Merk) with a gradient from 0 to 1 M KCl in lysis buffer. After dialysis at 4 °C against 10 mM KP, pH 7.5, 50 mM KCl, and 2 mM dithiothreitol, the pooled fractions were applied to a hydroxyapatite column (Merk) at 25 °C and eluted with a gradient from 0.01 to 0.5 mM potassium phosphate buffer, pH 7.5. As a further purification step, size exclusion chromatography was performed using a Superdex 200 gel filtration column (GE Healthcare, München, Germany) at 4 °C with 20 mM Hepes/KOH, pH 7.5, 400 mM KCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol. After analysis by SDS-PAGE, the gels were Coomassie-stained. The peak fractions containing protein with a molecular mass of 73 kDa were pooled and stored at a concentration of 12 mg/ml in the buffer of the gel filtration at −80 °C. The identity of Trap1 was determined by Western blotting using a polyclonal Trap1 antibody provided by D. Toft (Mayo Clinic) and mass spectrometry. The concentration of Trap1 was determined according to Ehresman (46).

**Multiangle Light Scattering**

The size of Trap1 was analyzed using an high-performance liquid chromatograph equipped with a size exclusion column (10 × 300 mm, Superdex 200, GE Healthcare, München, Germany) and a static light scatter instrument DAWN HELEOS (Wyatt Technology, Santa Barbara, CA). 150 μg of Trap1 in 40 mM Tris/HCl, pH 7.5, 150 mM KCl, and 5 mM MgCl2 were injected at 25 °C at a flow rate of 0.5 ml/min. The scattered light was detected at 18 angles, and the data were analyzed using the software Astra 5.3.0.18. (Wyatt Technology).

**Analytical Ultracentrifugation**

Sedimentation velocity analysis was performed using a Beckman Coulter Proteome Lab XL1 analytical ultracentrifuge (Beckman, Krefeld, Germany) equipped with absorbance optics and interference detector using a Ti60 rotor. Three samples of Trap1 in 20 mM Hepes/KOH, pH 7.5, 400 mM KCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol were run simultaneously at 20 °C and detected using absorbance at 280 nm (A290) (1-cm path length). The protein concentration was chosen so that the protein samples exhibited absorptions of 0.25, 0.58, and 0.8 A units. The buffer was used as a blank reference. Data were col-
selected at $A_{280}$ every minute. Data analysis was performed using SEDFIT Version 94 and SEDPHAT Version 5.01 (47). Beyond this analysis, we analyzed the data using the program Sedanal with an A2-to-2A-model, which provides further information about the dissociation constant of a monomer-dimer equilibrium. The value of 10 nM thus derived for the monomer-dimer with an A2-to-2A-model, which provides further information this analysis, we analyzed the data using the program Sedanal 

SEDFIT Version 94 and SEDPHAT Version 5.01 (47). Beyond 

pH 7.5, 100 mM KCl, and 5 mM MgCl$_2$ by a NAP column at 

increasing the temperature by 0.5°C/min. The data analysis 

curve of Trap1 was measured in potassium phosphate buffer by 

Isothermal Titration Calorimetry 

Titration experiments were performed using a VP-ITC sys-

tem (MicroCal Inc., Northampton, MA). AMP-PCP and ADP were injected in 28 aliquots ($6 \times 5 \mu l$, $19 \times 10 \mu l$, and $3 \times 20 \mu l$) of 400 mM into 1.424 ml of Trap1 (40 mM) at 25°C. RA was injected in 28 aliquots of 10 mM of 200 mM into Trap1 at 15 mM. All solutions contained 40 mM Hepes/KOH, pH 7.5, 150 mM KCl, and 5 mM MgCl$_2$. The resulting data were fit after subtracting the heats of dilution as described (25, 51) using the program Origin and the add-on for ITC of version 5.5 (MicroCal Software Incorporation Northampton, USA). Heats of dilution were determined in separate experiments from addition of ligands into buffer. Titration data were fit using a nonlinear least-square curve-fitting algorithm with three floating variables: stoichiometry, binding constant ($K_B = 1/K_D$), and change of enthalpy of interaction ($\Delta H^\circ$). The data for AMP-PCP binding are close to the lower limits of detection of ITC and consequently have large relative errors.

Fluorescence Measurements

Fluorescence measurements were performed with a Fluorolog fluorospectrometer (Horiba Jobin Yvon, Edison, NJ). The excitation wavelength was 296 nm, and the emission wavelength was 350 nm. The temperature of the cuvette was 25 °C. 1 mM Trap1 in 40 mM Hepes/KOH, pH 7.5, 150 mM KCl, and 5 mM MgCl$_2$ was investigated, and the spectra of three measurements were averaged.

ATPase Cycle of Trap1

ATPase Measurement

Coupled Colorimetric Assay—ATPase assays were performed as described earlier using an ATP-regenerating system (52). All experiments were carried out in 40 mM Hepes/KOH, pH 7.5, 150 mM KCl, 5 mM MgCl$_2$ at 25 °C using 5 μM Trap1, if not indicated otherwise. Hydrolysis was monitored over 20 min or until the absorption of NADH dropped below 0.3. The $K_{cat}$ value was determined using different ATP concentrations (between 0 and 200 μM). The assay was evaluated using GraFit Version 5.0.13 (Erithacus Software Limited, Horeley, UK). Inhibition of ATPase activity by RA was achieved by addition of 10 μM final concentration of the inhibitor.

Single Turnover—The ATPase activity of Trap1 was investi-
gated using 62 μM Trap1 and 52 μM ATP in 40 mM Hepes, pH 7.5, 150 mM KCl, and 5 mM MgCl$_2$ at 25 °C. For determination of the hydrolysis rate, the enzymatic hydrolysis of 16 μl of the nucleotide-protein-complex was stopped by denaturing with 4 μl 50% trichloroacetic acid at 4 °C. After centrifugation at $10,000 \times g$, 10 μl of the supernatant was neutralized by adding 20 μl of 2 M KOAc. The nucleotides were analyzed via a C18 reversed phase column (ODS Hypersil, 5 μm 250 × 4 mm, Bischoff, Leonberg, Germany) using a high-performance liquid chromatogram from Waters (Milford, MA), 50 mM KPi, pH 6.8, and a flow rate of 1.5 ml/min. 10 μl of the neutralized mixture were applied, the nucleotides were detected at an absorption of 254 nm, and the area of the single peaks was integrated. An experiment under the same conditions with free nucleotide showed no spontaneous hydrolysis. After calibration with a nucleotide standard consisting of ATP, ADP, and AMP at 10 μM each, the amount and ratio of ATP to ADP was calculated.

Transient Kinetics

Stopped-flow measurements were performed with a HiTech SF-61 DX2 instrument in 40 mM Hepes/KOH, pH 7.5, 150 mM KCl, and 5 mM MgCl$_2$ at 25 °C. The excitation slit was set to 0.5 nm, if not indicated otherwise. Nucleotide binding reactions were monitored by intrinsic tryptophan fluorescence upon excitation at 296 nm with a long pass filter (cutoff of 320 nm). In the case of the fluorescent ATP analog (Pγ)-MABA-ATP, binding reactions were followed either by fluorescent energy transfer between tryptophan and the bound (Pγ)-MABA-ATP upon excitation at 296 nm or by direct excitation of the fluorescent MABA group at 364 nm. In both cases the emission was detected using a cutoff filter of 420 nm.

Each experiment was performed between three and eight times, and the resulting time traces were averaged with the software from HiTech Scientific. Concentrations are noted as conc1/conc2 (syringe/cell).

The primary binding data of the stopped-flow measurements were analyzed with either a double-exponential equation for ATP (Equation 1), or a single-exponential equation (Equation 2), for ADP, AMP-PCP, and (Pγ)-MABA-ATP binding, displacement, and competition experiments.

\[
F = F_0 + A_1(1 - e^{-kt}) + A_2(1 - e^{-kt}) \quad (\text{Eq. 1})
\]

\[
F = F_0 + A_1(1 - e^{-kt}) \quad (\text{Eq. 2})
\]
**ATPase Cycle of Trap1**

In Equations 1 and 2, $F$ is signal, $A$ is amplitude, $F_0$ is offset, and $k$ is observed rate constant. Most of the data indicated one additional minor phase. This additional phase was always independent of the nucleotide concentration and thus suggested photobleaching. This conclusion was confirmed by repeating the experiments with an auto-shutter, which abolished this phase. We therefore extended data analysis with either an additional linear or exponential term to allow for appropriate correction of the drift but omitted it in the discussion of data, because it is not relevant for any conclusions drawn.

Considering the fact that the measurements of ADP, AMP-PCP, and (Pγ)-MABA-ATP with Trap1 were performed under pseudo first order conditions (one ligand in excess) and assuming a simple one-step binding mechanism, the data were analyzed by $k_{obs} = k_{on} \times [L]_{excess} + k_{off}$, with $[L]_{excess}$ being the concentration of ligand in excess and therefore a plot of $k_{obs}$ versus ligand concentration gives $k_{off}$ as the intercept and $k_{on}$ as the slope. The secondary data of ATP binding experiments, observed rate constants as obtained from a double exponential fit of each time trace versus concentration of ligand in excess, were then analyzed as described previously (53). For a two-step binding mechanism (see Fig. 7), the microscopic rate constants can be determined via $k_1 \times k_2 = k_{1, on} \times [ATP] + k_{1, off} + k_{2, on} + k_{2, off}$, where $k_1$ is the observed rate constant, $k_{on}$ is the association rate constant, and $k_{off}$ is the dissociation rate constant. The product of the observed rate constants of the two phases versus concentration of ligand is also analyzed with a linear fit. The mechanism deduces that $k_1 \times k_2 = k_{1, on} \times (k_{2, on} + k_{2, off}) \times [ATP] + k_{1, off} \times k_{2, off}$. The four rate constants are then determined as follows,

$$k_{1, on} = \text{slope}(1)$$  \hspace{1cm} (Eq. 3)

$$k_{1, off} = \frac{\text{int}(1) - \text{slope}(2)}{\text{slope}(1)}$$  \hspace{1cm} (Eq. 4)

$$k_{2, on} = \frac{\text{int}(2)}{k_{1, off}}$$  \hspace{1cm} (Eq. 5)

$$k_{2, off} = \text{int}(1) - k_{1, off} + k_{2, off}$$  \hspace{1cm} (Eq. 6)

where int(1) and int(2) are the intercepts of the $k_1 + k_2$ and $k_1 \times k_2$ replots, and slope(1) and slope(2) are the corresponding slopes.

The rate constants for binding and release of unlabeled nucleotides were also determined by displacement measurement where ATP was added in various concentrations to a preformed complex of (Pγ)-MABA-ATP with Trap1. Applying $k_{off}$ and $k_{on}$ known from pseudo-first order experiments as described above the values for unlabeled ATP were determined using the following adapted equations for dissociative mechanisms (54),

$$k = \frac{1}{2}(a_1 + a_2 + a_3)$$  \hspace{1cm} (Eq. 7)

$$a_1 = k_{on,1} \times (L_{10} - L_{E_0})$$  \hspace{1cm} (Eq. 8)

where $E_0$ and $L_{10}$ are the concentration of the components of the preformed complex, $L_{2o}$ is the competing ligand, and $k_{on}$ and $k_{off}$ are the association and dissociation rate constants of (Pγ)-MABA-ATP (constants and concentrations with index 1) and ATP (constants and concentrations with index 2). $K_1$ and $K_2$ define the state when the reactions are in equilibrium. It should be noted that $K_1$ is the association constant of (Pγ)-MABA-ATP and $K_2$ is the dissociation constant of ATP. $E_0$ is the concentration of free enzyme at a certain concentration of ligand in excess, and $K_{off}$, $K_{on}$ are the affinity determined independently with a pseudo-first order model (Kuzmic, BioKin Co., Pullman, WA) (55). The underlying model is depicted in Fig. 7. Data obtained from pre-steady state kinetic experiments as well as steady-state and single turnover...
ATPase data were analyzed simultaneously with the relevant kinetic parameters being shared by all data sets. Response parameters were shared within a data set except for the effects of photobleaching, which were fit locally for each individual data set. To check whether all parameters are constrained sufficiently by the experimental data available a sensitivity analysis was performed using the program Dynafit (Kuzmič, BioKin), which confirmed that all parameters converged to well defined minima.

RESULTS

Characterization of Trap1—Recombinant human Trap1 lacking the first 59 amino acids (the mitochondrial signal sequence) was overexpressed in E. coli and purified to homogeneity (see “Experimental Procedures”). The calculated molecular mass of 73,547 Da was verified by matrix-assisted laser desorption ionization mass spectrometry. To assess the oligomeric state of purified Trap1, analytical ultracentrifugation was performed as described under “Experimental Procedures.” The sedimentation velocity experiment showed that the protein is dimeric (Fig. 1). This result was confirmed by multiangle light scattering (data not shown). To further estimate the structural integrity and thermal stability, CD spectra were recorded in the far-UV wavelength region. The shape of the spectrum indicated that the secondary structure has a high \( \alpha \)-helical content that was not significantly changed upon nucleotide or RA binding (data not shown). The midpoint of the melting curve of Trap1, as monitored by CD spectroscopy, was 55 °C at a concentration of 2 \( \mu \)M (data not shown). Because the transition is not reversible, no thermodynamic data could be derived from the melting curve.

Nucleotide Binding Properties Determined by ITC—The affinities and stoichiometries of ADP, AMP-PCP, and RA for Trap1 were determined by ITC. As depicted in Fig. 2, ADP binds with low affinity to Trap1. The dissociation constant of 35 \( \mu \)M is in agreement with the observation that purified Trap1 is in the nucleotide-free state. The results for AMP-PCP and RA (Fig. 2) are listed in Table 1. In the case of AMP-PCP, the affinity of 109 \( \mu \)M is at the upper detection limit of the method. The affinities for AMP-PCP and RA were determined to be 109 and 0.025 \( \mu \)M. The values are reminiscent of values determined previously for nucleotides to other Hsp90 analogs (41). The stoichiometry for all nucleotides and the inhibitor is close to one ligand per Trap1 monomer.

ATP Hydrolysis—Members of the Hsp90 family are characterized by an intrinsic slow ATPase activity. In principle, low ATPase activity can be the result of highly active ATPases contaminating the protein preparation. For Trap1, it had been reported that the ATPase activity could be completely suppressed by the Hsp90-specific inhibitor RA (12, 37). In agreement with this report, the addition of RA to our Trap1 preparations resulted in a complete inhibition of ATPase activity (see below).

To dissect the ATPase cycle, we investigated the hydrolysis using two approaches: steady-state and single turnover experiments (see “Experimental Procedures”). Measurements performed under steady-state conditions (Fig. 3A) followed simple Michaelis-Menten kinetics. The data imply a hydrolysis rate of 0.0027 s\(^{-1}\) (= 0.16 min\(^{-1}\)) with a \( K_m \) value of 10.5 \( \mu \)M. The temperature dependence of the ATPase activity of Trap1 was tested with 200 \( \mu \)M ATP in a range between 25 °C and 60 °C (Fig. 3B). The hydrolysis rate increased between 25 °C and 55 °C ~ 200-fold, which corresponds to the high activation energy of 152 kJ/mol. The rate reaches a maximum at 55 °C where the protein starts to denature (see Fig. 3B). Above this temperature, the protein lost its activity during incubation completely and irreversibly, in agreement with a melting temperature of 55 °C.

To test whether hydrolysis or release of ADP is the rate-limiting step of the ATPase cycle, ATPase activities were recorded under single turnover conditions. For this assay, we mixed Trap1 and ATP in a ratio of 1:0.8 at a concentration \( [E] \gg K_d \) to ensure true single turnover conditions. The conversion of ATP to ADP followed a single exponential equation.
ATPase Cycle of Trap1

![Image](125x452)

**FIGURE 2. Determination of ligand affinities by ITC.** A. 403.7 nM ADP was titrated into the cell containing 39.2 µM Trap1 at 25 °C. A1 shows the change of heat during the titration. A2 depicts the heat of dilution and fitted data. The resulting affinity is 35.0 µM with a stoichiometry of 1:1. B, the inhibitor RA was titrated at a concentration of 200 µM into 14.41 µM Trap1 (K_D = 25.3 nM) with B1 depicting the change of heat during titration and B2 the fitted data.

| Ligand   | K_D (µM) | n     | ∆H (kJ/mol) | ∆S (J/molK) |
|----------|----------|-------|-------------|-------------|
| ADP      | 35.0 ± 3.7 | 0.90 ± 0.05 | -12.4 ± 1.0 | -212         |
| AMP-PCP  | 109 ± 42.0 | 1.39 ± 0.4 | -30.5 ± 15.2 | -840         |
| RA       | 0.025 ± 0.004 | 1.58 ± 0.01 | -8.6 ± 0.1 | 5.9          |

yields a rate constant of 0.002 s^{-1} (=0.12 min^{-1}) (Fig. 3C), which is comparable to the hydrolysis rate (k_{hyd}) obtained under steady-state conditions. This indicates that hydrolysis, or an undetected conformational change preceding it, is the rate-limiting step of the cycle. One important question concerning the ATPase cycle is whether ATP is trapped after binding and thus committed to hydrolysis. The answer to this question would also tell us whether Trap1 is predominantly in an open or closed state during steady-state hydrolysis. So far two different results have been obtained for Hsp90 proteins. For cytosolic Hsp90 from yeast (40), commitment was detected, whereas endoplasmic Grp94 was found predominantly in the open conformation even after ATP binding (42). To test the commitment, we performed an experiment under single turnover conditions and stopped the hydrolysis of ATP with the addition of excess RA. This prevented ATP rebinding instantaneously, due to its significantly higher affinity (see Table 2) and thus constituted an effective chase of the Hsp90-ATP complex. In the case of commitment, hydrolysis was expected to continue until completion, whereas, when ATP was not trapped, hydrolysis should immediately stop upon addition of the inhibitor. We observed that the latter is the case for Trap1: when RA was added, the ATPase activity stopped within seconds (Fig. 3C). This observation can be explained by two different scenarios. Either the protein remains mainly in the open conformation after ATP binding, or the reopening of the closed state is faster than the actual hydrolysis. This question was addressed further by rapid kinetic studies.

**Analysis of Nucleotide Binding Using Transient Kinetics**—To test whether it is also possible to detect nucleotide binding by changes in the intrinsic fluorescence of Trap1, we monitored the emission spectra in the presence and absence of ligands. In the presence of ligands, the fluorescence intensity decreased by one-third without a significant shift in the peak emission (data not shown). To analyze the binding of ligands and the subsequent conformational changes in more detail, we performed transient kinetics measurements (see “Experimental Procedures”). The mixing of ATP and Trap1 resulted in a decrease of the tryptophan fluorescence that could be described with a double exponential equation (Fig. 4A). Fig. 4B depicts the observed rate constants versus ATP concentration from the double exponential fits of the time traces. Even though the rate constants of the second phase are low compared with the ones for the first phase, Fig. 4C indicates that both phases contribute significantly to the fluorescence decrease. These data allow for the first time to kinetically resolve the conformational changes for an Hsp90 family member upon binding of ATP. The plots of product and sum of the observed rate constants versus concentration of ATP (Fig. 4D) are used to extract the microscopic rate constants of the two steps as described under “Experimental Procedures.” This analysis yielded values for the rate constants of association and dissociation for ATP of 0.03 ± 0.001 µM^{-1}s^{-1} and 1.0 ± 0.3 s^{-1}, respectively, as well as the rate constants for closing and opening of the nucleotide binding pocket of 0.076 ± 0.041 and 0.034 ± 0.09 s^{-1}, respectively (see “Experimental Procedures”). The affinity of ATP for Trap1 in the “apparent” collision complex was calculated using the rates for binding and release (as described under “Experimental Procedures”) and yielded a value of 34.3 ± 9.1 µM. The position of the equilibrium of the conformational change of 0.45 was calculated analogously, indicating that Trap1 is predominantly in the closed conformation (70%), but the velocity of reopening the nucleotide binding pocket is 10-fold faster than the ATPase activity or a step preceding it. It should be noted that an additional experiment with very high ATP concentrations (up to 5 mM) indicated that the first step of ATP binding as described here as one step, can actually be resolved into two steps as was
ATPase Cycle of Trap1

FIGURE 3. ATPase activity of Trap1. A, determination of the Michaelis constant (Km) of Trap1 for ATP. 5 μM human Trap1 was mixed under steady-state conditions with increasing concentration of ATP (up to 200 μM final concentration). This resulted in a K_m of 10.5 μM and k_{off} = 0.0027 s^{-1}. Although the substrate concentration (ATP) is not much higher than the enzyme the steady-state approximation still holds due to the ATP regenerating properties of the coupled assay (see “Experimental Procedures”). B, temperature dependence of Trap1 ATPase activity. 5 μM Trap1 was incubated under steady-state conditions at different temperatures. Arrhenius plot (inset of Fig. 3B) of the dependence of ATPase activity on the temperature follows a straight line. The ATPase activity increases ~200-fold between 25 and 55 °C, anticipated before for human Hsp90 (41). This follows from the fact that the apparent rate constant of the first phase versus [ATP] follows a hyperbola with a K_D of 1900 μM for the first fast step (presumably true collision complex formation) and a second step with ~100 s^{-1} and 0.9 s^{-1} for the forward and backward reactions, respectively (data not shown). Even though the detection of this conformational change is highly interesting, we decided to not include this step in our further analysis and discussion due to several reasons: 1) further conclusions for the complete ATPase cycle will not be affected by this extension, 2) a global fit including this data set was impossible to perform due to inner filter effects at high ATP concentrations and thus amplitude problems, and 3) most importantly, comparisons with studies of the other Hsp90 variants investigated so far could otherwise not be drawn directly. However, the analysis is shown in full detail in the supplemental materials.

To analyze the binding kinetics of the hydrolysis product, we then carried out similar measurements using ADP as binding partner. The observed rate constants versus concentration of ADP (data not shown) followed a straight line resulting in a k_{on} and k_{off} of 0.04 ± 0.002 μM^{-1} s^{-1} and 1.6 ± 0.3 s^{-1}, respectively, and a K_D of 38.7 ± 7.6 μM. This indicates that ADP binding can be described by a one-step mechanism. The amplitudes of the monitored fluorescence decrease for ADP correspond to the sum of the amplitudes determined for ATP binding. This indicates that also ADP binding induces a compact conformation of Trap1, but the corresponding structural rearrangements cannot be resolved with the stopped-flow mixing method used here. Furthermore, the amplitudes contain information about the dissociation constant at equilibrium conditions. The data were analyzed with a quadratic equation (see “Experimental Procedures”), and a dissociation constant of 24.8 μM was calculated. Thus, unlike yeast and human Hsp90 (40, 41), but similar to Grp94 (42), Trap1 seems to bind ATP and ADP with equal affinities (Table 2). Surprisingly, AMP-PCP binds significantly weaker (Table 2). Unlike ATP, AMP-PCP binding can be described by a one-step mechanism indicating that the ATP analog does not induce conformational changes.

To substantiate the kinetic parameters obtained by the measurements using tryptophan fluorescence as a signal, which can be ambiguous, we investigated the affinity and kinetics of Trap1 and ATP by an independent method. We used the ATP analog (Pγ)-MABA-ATP, which changes its fluorescence upon binding to Hsp90 (40, 42). Fig. 5A depicts the time traces as monitored after mixing of (Pγ)-MABA-ATP and Trap1 in a stopped-flow instrument. The fluorescence of (Pγ)-MABA-ATP increases upon binding to Trap1 due to the change in the environment of the fluorescence label in the hydrophobic binding pocket of Trap1. The time traces follow a mono-exponential fit. The affinity of Trap1 above 55 °C Trap1 denatures and loses its ATPase activity. The slope indicates an activation energy of 152.1 kJ mol^{-1}.
for (Pγ)-MABA-ATP was determined analyzing the binding isotherms (Fig. 5B) of the primary curves with the quadratic equation (see "Experimental Procedures") yielding a $K_D$ of 8.4 μM. The kinetic parameters of the binding were derived from a rate constant versus concentration of Trap1 replots. The data followed a straight line as depicted in the inset of Fig. 5B. Because the experiment was performed under pseudo-first order conditions, the rate constants $k_{on}$ and $k_{off}$ were determined to be 0.035 ± 0.0004 μM$^{-1}$s$^{-1}$ and 0.34 ± 0.004 s$^{-1}$, respectively. The resulting $K_D$ ($=k_{off}/k_{on}$) of 9.9 μM is in good agreement with the affinity obtained by analyzing the binding isotherm. Further, we performed a titration experiment mixing increasing concentrations of (Pγ)-MABA-ATP with a constant concentration of Trap1 (data not shown). In this case, the background fluorescence increased significantly when excited directly, because not only the bound but also the free fluorescent nucleotide analog contributed to the detected signal. Thus, when titrating the ligand, the fluorescence of (Pγ)-MABA-ATP was detected via fluorescent energy transfer fluorescence upon excitation of tryptophan to ensure that only bound nucleotide is excited and that unbound nucleotide does not contribute to the total fluorescence monitored. The values obtained in this experiment are in good agreement with the previously described experiment (Table 2). These experiments were performed under pseudo-first order conditions. Thus, the observed association rate constant will be determined by the binding partner in excess. The observation that the variation of (Pγ)-MABA-ATP as well as of Trap1 leads to the same association rate constant indicates that the two binding partners are indeed binding with a 1:1 stoichiometry. Otherwise the determined association rate constants would deviate according to the effective concentration of the binding partner in excess.

With the knowledge of the kinetic parameters of (Pγ)-MABA-ATP, we performed displacement measurements to determine the values for ATP, independent from tryptophan fluorescence. A preformed complex consisting of Trap1 and (Pγ)-MABA-ATP was mixed in the stopped-flow instrument with ATP (see "Experimental Procedures"), and the decreasing fluorescence upon fluorescent energy transfer excitation was monitored (Fig. 6A). The time traces, which followed a single exponential fit, were analyzed according to the equation for a dissociative mechanism (see "Experimental Procedures") using the known rate constants for association and dissociation of (Pγ)-MABA-ATP. This analysis resulted in $k_{on}$ and $k_{off}$ values of 0.03 μM$^{-1}$s$^{-1}$ and 0.81 s$^{-1}$ for unlabeled ATP, respectively, which give a $K_D$ of 29.9 μM for ATP. This corresponds to the parameters obtained by monitoring tryptophan fluorescence for the first step. The binding isotherms of the time traces were analyzed using a cubic equation (Fig. 6C) applying the $K_D$ of (Pγ)-MABA-ATP obtained by direct binding measurements as described above (see “Experimental Procedures”). The $K_D$ determined by this approach was 16.5 μM, which is comparable to the affinity obtained by the other approaches (see Table 2). In summary, from these results we can conclude that (Pγ)-MABA-ATP monitors the first step of ATP binding only and is thus obviously not able to accomplish the conformational change induced by ATP.

### Table 2

| Nucleotide/thermodynamic | $K_D$ (μM) |
|---------------------------|------------|
| ADP                       | 3.5 ± 0.2 (0.28 ± 0.03) |
| AMP                       | 0.03 ± 0.0004 (0.00001) |
| ATP                       | 0.03 ± 0.001 |
| ATP-competition           | 0.03 ± 0.001 |

The kinetic parameters of the binding were derived by direct binding experiments as described under "Experimental Procedures". The values obtained in this experiment are in good agreement with the previously described experiment (Table 2). These experiments were performed under pseudo-first order conditions. Thus, the observed association rate constant will be determined by the binding partner in excess. The observation that the variation of (Pγ)-MABA-ATP as well as of Trap1 leads to the same association rate constant indicates that the two binding partners are indeed binding with a 1:1 stoichiometry. Otherwise the determined association rate constants would deviate according to the effective concentration of the binding partner in excess.

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Performing analogous measurements to investigate the affinity of RA (data not shown) indicated strong inner filter effects of RA, and therefore amplitudes had to be corrected for the absorption of light by RA. The obtained values correspond to those obtained independently by ITC measurements and are listed in Table 2. The comparison of the binding and release rate constants of RA with those of nucleotides indicates that the higher affinity of the inhibitor is caused by accelerated binding as well as by a reduced release rate constant.

Global Fit—To test the underlying mechanistic model for the ATPase cycle of Trap1 in a comprehensive manner, we applied a global fit approach using the program DYNAFIT (55). This analysis is a valuable tool to check for consistency of the proposed mechanism. Furthermore, because it simultaneously processes information of all signal amplitudes, it also has the potential to detect steps that are unnoticed otherwise, specifically with the typical pseudo-first order analyses. To that end, we included stopped-flow data of ATP and ADP binding to Trap1 as well as data from single turnover and steady-state ATPase activity experiments. In the fits described here, all rate constants relevant for the mechanism (Fig. 7) as derived from individual experiments were used as adjustable parameters. The global fit procedure resulted in rate constants for the individual steps (listed in Table 2), which were similar to the ones obtained by our previous pseudo-first order analyses of the individual experiments (see Fig. 7). The consistency between the parameters obtained by the different analyses confirms our proposed model for the ATPase cycle. The rate constants for the binding of the nucleotides to Trap1 as well as the kinetics of the conformational change are well determined by the global fit. The sensitivity analysis shows that all parameters exhibit well defined minima. A point to be considered is the three times increased hydrolysis rate when determined by global fit analysis. A deviation in this range can be caused by the complexity of the system and does not necessarily indicate a missing step in the mechanism. To ensure that this is indeed the case, we analyzed our data using models with either one step less or an additional step before hydrolysis. Comparisons of the mean squares and the residuals of the data sets fitted to the different models confirm the proposed model. When we describe ATP binding by one step only the mean square value increases by almost 300% (0.006 compared with 0.0021 for...
the proposed model, and residuals show pronounced systematic deviations. Addition of a further step prior to hydrolysis neither reduces the mean square significantly (ca. 6%) nor improves the quality of the residuals. This confirms that we considered the minimal amount of steps necessary to describe the system sufficiently. Therefore, according to Occam’s razor, the kinetic model presented here for the Trap1 ATPase cycle is the most plausible one for the given data sets.

**DISCUSSION**

Trap1 forms a tight dimer with a melting temperature of 55 °C. The intrinsic stability is similar to that of cytosolic Hsp90 from yeast (42) but remarkably different from the other organelle-based Hsp90 studied so far, namely endoplasmic Grp94, which denatures already slightly above 30 °C (42). Contrary to Grp94 (42), Trap1 shows a highly pronounced activation under heat shock conditions with a 200-fold increase of ATPase activity between 25 and 55 °C.

Along with differences in ATP binding properties due to variations between the N-terminal and middle domains of yeast Hsp90 (44), *E. coli* HtpG (26), and canine Grp94 (48) as evidenced by their crystal structures, the affinities of Hsp90 enzymes for nucleotides vary in a wide range between 3 and 500 μM (Table 3). Using the intrinsic tryptophan fluorescence of Trap1, we found that nucleotides bind with $K_D$ values of 34.3, 38.7, and 85.9 μM for ATP, ADP, and AMP-PCP, respectively (Table 2). These data were confirmed independently by ITC measurements (Table 1). Both data derived from kinetic stopped-flow experiments and ITC imply that the non-hydrolysable ATP analog AMP-PCP binds significantly weaker to Trap1 than ATP. Transient kinetic studies further showed that AMP-PCP binding does not induce conformational changes in Trap1. This suggests that the bridging oxygen between the β- and γ-phosphate is important for domain closure. In agreement with Grp94 (42), Trap1 binds approximately ten times tighter to ATP then yeast Hsp90 (40) and human Hsp90 (41).

Whereas nucleotides bind to Trap1 in the low micromolar range, RA shows tight binding with a $K_D$ of 25 nM based on ITC and kinetic measurements confirming its ability to efficiently chase nucleotides from Trap1. Interestingly, the binding of RA is also entropically driven, possibly an indication that here more well positioned water molecules are released from the active site.

The kinetics of association of all ligands measured with Trap1 are in a range from 0.03 to 0.04 μM$^{-1}$s$^{-1}$. This is one magnitude lower than reported for human Hsp90 (41), yeast Hsp90 (40), and Grp94 (42).

An important step of the ATPase cycle is the hydrolysis of ATP. Up to now, the ATPase cycles of human Hsp90 (41), yeast Hsp90 (40), and Grp94 (42) were investigated in some detail by kinetic approaches. The reported rate constants for ATP hydrolysis of 0.0017 s$^{-1}$ (0.1 min$^{-1}$) at 30 °C for Trap1 (12) was confirmed by our experiments, with a slightly higher value of 0.0027 s$^{-1}$ (0.16 min$^{-1}$) at 25 °C in our hands. This observed ATPase activity is relatively low compared with other ATPases but comparable to the rates measured for other Hsp90 (40, 41). Addition of the Hsp90-specific inhibitor RA resulted in a complete suppression of ATPase activity suggesting that there were
For yeast Hsp90 it was shown that ATP binding results in a conformational change of the enzyme. Again, this change that could be kinetically resolved by the mixing experiments implies that in the presence of ATP the predominant fraction of Trap1 is in the closed conformation (70%), comparable to the results for yeast Hsp90 (80%).

In summary, the ATPase cycle of Trap1 exhibits interesting differences compared with the known Hsp90 ATPase cycles. ATP binding leads to a spectroscopic signal that allowed describing the essential conformational change kinetically for the first time for a member of the Hsp90 family. Despite this difference, the ATPase mechanisms of all Hsp90 members investigated so far appear to be very similar, at first glance. The binding of ligands, mainly nucleotides, all appear with comparable rate constants (Table 3). Besides ATP, no other nucleotide appears to advance beyond formation of the collision complex. Unlike ATP, neither ADP, AMP-PCP, (Pγ)-MABA-ATP, nor inhibitors like RA induced a visible conformational change that could be kinetically resolved by the mixing methods used here.

Concerning the balance of this open/closed state in the presence of ATP two major classes of Hsp90 seem to appear. They are either predominantly in the closed state in the presence of ATP (yeast Hsp90, human Hsp90, and Trap1) (40–42) or in the open state (Grp94) (42). If they do close, the kinetic ratios of the open/close process versus hydrolysis determine commitment for ATP hydrolysis. The particular relation of these different balances and kinetics of the key steps in the ATPase cycle have to be determined yet, specifically also in the context of their regulation co-chaperones.
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