Kinetic Characterization of the ATPase Cycle of the Molecular Chaperone Hsc66 from Escherichia coli*

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Hsc66 from Escherichia coli is a constitutively expressed hsp70 class molecular chaperone whose activity is coupled to ATP binding and hydrolysis. To better understand the mechanism and regulation of Hsc66, we investigated the kinetics of ATP hydrolysis and the interactions of Hsc66 with nucleotides. Steady-state experiments revealed that Hsc66 has a low affinity for ATP (K\textsuperscript{ATP} = 12.7 μM) compared with other hsp70 chaperones. The kinetics of nucleotide binding were determined by analyzing changes in the Hsc66 absorbance spectrum using stopped-flow methods at 23 °C. ATP binding results in a rapid, biphasic increase of Hsc66 absorbance at 280 nm; this is interpreted as arising from a two-step process in which ATP binding (k\textsuperscript{ATP} = 4.2 × 10\textsuperscript{5} M\textsuperscript{−1} s\textsuperscript{−1}, k\textsubscript{dATP} = 1.1 s\textsuperscript{−1}) is followed by a slow conformational change (k\textsubscript{cont1} = 0.1 s\textsuperscript{−1}). Under single turnover conditions, the ATP-induced transition decays exponentially with a rate (k\textsubscript{decay} = 0.0013 s\textsuperscript{−1}) similar to that observed in both steady-state and single turnover ATP hydrolysis experiments (k\textsubscript{nyst} = 0.0014 s\textsuperscript{−1}). ADP binding to Hsc66 results in a monophasic transition in the absence (k\textsubscript{ADP} = 7 × 10\textsuperscript{5} M\textsuperscript{−1} s\textsuperscript{−1}, k\textsubscript{dADP} = 60 s\textsuperscript{−1}) and presence of physiological levels of inorganic phosphate (k\textsubscript{ADP(Pi)} = 0.28 × 10\textsuperscript{5} M\textsuperscript{−1} s\textsuperscript{−1}, k\textsubscript{dADP(Pi)} = 9.1 s\textsuperscript{−1}). These results indicate that ATP hydrolysis is the rate-limiting step under steady-state conditions and is >10\textsuperscript{3}-fold slower than the rate of ADP/ATP exchange. Thus, in contrast to DnaK and eukaryotic forms of hsp70 that have been characterized to date, the R ⇔ T equilibrium balance for Hsc66 is shifted in favor of the low peptide affinity T state, and regulation of the reaction cycle is expected to occur at the ATP hydrolysis step rather than at nucleotide exchange.

Hsp70 proteins comprise a ubiquitous family of ATP-dependent molecular chaperones that have been shown to play roles in stress responses, protein processing, and protein folding (for reviews see Refs. 1–6). To accomplish their cellular roles, Hsp70 proteins couple nucleotide binding and hydrolysis with conformational changes that control their peptide substrate affinity. ATP binding results in a conformational change leading to the formation of a tense state (T state) with reduced affinity for peptide substrates, and subsequent hydrolysis to ADP and phosphate relieves this tense state and results in formation of a relaxed state (R state) with increased peptide binding affinity (7–11).

DnaK from Escherichia coli has been the prototypical hsp70 family member for elucidating the mechanism of hsp70 chaperone action. Kinetic studies have shown that in the absence of cochaperones and substrates, the rate of interconversion between the R and T states of DnaK is slow on the time course of physiological processes with both ATP hydrolysis and ADP/ATP exchange occurring with rates ≤ 1 min\textsuperscript{−1} (11–13). Both of these kinetic steps in the DnaK ATPase cycle, however, are subject to regulation by cochaperones; the rate of ATP hydrolysis is stimulated up to ~10\textsuperscript{3}-fold by DnaJ (14–17), and the rate of ADP/ATP exchange is stimulated up to ~10\textsuperscript{3}-fold by GrpE (17).

Many bacteria contain a second constitutively expressed hsp70, in addition to DnaK, encoded by the hscA gene and designated Hsc66 (heat shock cognate, Mr ~66,000; see Refs. 18 and 19). Although the exact cellular role of Hsc66 has not been determined, localization of hscA to a gene cluster (iscSUA-hscBA-fdx) encoding proteins thought to be involved in the assembly of iron-sulfur clusters (20) suggests a specialized role in the folding of Fe/S proteins. In vitro studies indicate Hsc66 exhibits chaperone activity as evidenced by its ability to prevent the aggregation of model polypeptide substrates (21). In addition, Hsc66 exhibits slow, intrinsic steady-state ATPase activity (~1 min\textsuperscript{−1} at 37 °C and <0.1 min\textsuperscript{−1} at 20 °C; Ref. 22), and ATP causes effects consistent with destabilization of Hsc66-peptide complexes (21). These results support a model for Hsc66 action similar to that proposed for DnaK in which the rates of peptide binding and release are coupled to ATP hydrolysis and ADP/ATP exchange rates.

In contrast to DnaK, however, the ATPase activity of Hsc66 is not affected by physiological levels of DnaJ and GrpE (21). Instead, Hsc66 is regulated by a specific, 20-kDa cochaperone encoded by the hscB gene and designated Hsc20 (21, 22). The mechanism whereby Hsc20 stimulates the ATPase activity of Hsc66, however, is not known, and kinetic characterization of the Hsc66 ATPase reaction cycle will be required to address this question. The finding that Hsc66 does not interact with the nucleotide exchange protein GrpE also raises the question of whether the rates of ADP and ATP exchange with Hsc66 differ significantly from those of DnaK and other GrpE-regulated hsp70 chaperones.

To understand better the kinetics of Hsc66 and nucleotide interactions and to provide a framework for future evaluation of regulation of its activity, we have carried out a kinetic characterization of the Hsc66 ATPase reaction cycle. Our results indicate that ATP hydrolysis is the rate-limiting step under steady-state conditions and is slow compared with the rate of ADP/ATP exchange. Thus, in contrast to DnaK, in which there is a balance between the R and T states in the absence of auxiliary factors, the R ⇔ T equilibrium balance for Hsc66 is shifted in favor of the T state. Cochaperone stimulation of Hsc66, therefore, is not likely to be exerted on nucleotide exchange but is instead expected to be on the rate-determining ATP hydrolysis step.
Kinetic Analysis of the Hsc66 ATPase Reaction Cycle

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—Recombinant Hsc66 was expressed and purified as described previously (22). The concentration of Hsc66 was measured spectrophotometrically using a calculated molar extinction coefficient of 19,600 (cm·M⁻¹) determined using average absorbivities for tryptophan and tyrosine of 5,600 and 1,400 (cm·M⁻¹) (23–25).

Absorption spectra of purified Hsc66 indicated that no bound nucleotides were present as previously reported (22).

**Steady-state ATPase Activity of Hsc66**—Reaction mixtures (100 μl) contained HKM buffer (100 mM HEPES, pH 7.5, 150 mM KCl, and 10 mM MgCl₂), 1 μM Hsc66, 0.1–1 μCi of [α-³²P]ATP (Amersham Pharmacia Biotech), and 2–125 μM ATP (Sigma). Each reaction was incubated without ATP at 23 °C for 5 min prior to initiating the hydrolysis reaction. Aliquots of the reaction mixture (10 μl) were removed at various times after ATP addition and quenched by adding 10 μl of acetic acid (Fisher). The sample was mixed thoroughly and centrifuged at 2,000 × g for 30 s, and 3 μl was spotted on polyethylene-cellulose TLC sheets (Supelco). TLC sheets were dried and developed in 1 M formic acid containing 0.5 μL LiCl, and the fraction of ADP present was determined using a PhosphorImager (Molecular Dynamics). The rate of hydrolysis (Vₜ) was determined for each concentration of ATP by linear regression analysis, and the fraction of ATP hydrolyzed was not allowed to exceed 20%. As found for other hsp70 proteins (28–30), no ATPase activity was observed for Hsc66 in the absence of potassium ions (data not shown), and a physiological concentration of potassium (150 mM) was included in all assay buffers. Kᵣ and V₉ₐᵣ₆ values were determined by fitting the Michaelis-Menten equation to a plot of Vₕ versus [ATP] using Kaleidagraph (Synergy Software) which makes use of the Levenberg-Marquardt algorithm (26) to approximate curve fits. The rates for Vₗ shown represent an average of two independent experiments with error bars corresponding to ±1 S.D.

**The Rate Constant for ATP Hydrolysis**—Reactions identical to those described for steady-state ATPase analyses, except Hsc66 was present in excess over ATP (150 μM Hsc66 and 1 μM ATP containing 0.1–1 μCi of [α-³²P]ATP), and ATP hydrolysis was followed for 90 min at 23 °C. Data were corrected for the background level of [α-³²P]ADP (typically ~2%) and an unknown impurity (typically ~2%) which migrated farther than ADP on TLC. The conversion of ATP to ADP was plotted versus time and fit to a first-order equation using Kaleidagraph. The data shown represent the average of two independent experiments with error bars corresponding to ±1 S.D.

**ATP Synthesis Experiments**—ATP synthesis reactions (600 μl) were performed in the presence and absence of 50% H₂¹⁸O and contained 50 mM ATP, pH 7.5, 150 mM KCl, 10 mM MgCl₂, and 100 μM Hsc66. After incubation at 37 °C for 48 h, D₂O was added to a final concentration of 20% to maintain a deuterium frequency lock and allow for optimization of the magnetic field homogeneity in ³¹P NMR experiments. One-dimensional ³¹P NMR spectra were recorded at 161.97 MHz on a Bruker (Billerica, MA) DRX400 spectrometer. Spectra were acquired at 23 °C in Fourier transform mode using a 4-μs pulse (33° flip angle) and a spectral width of 200 Hz. Two thousand repetitive scans were signal averaged, and exponential modulation was applied to the free-induction decay. Chemical shifts are reported with reference to 85% H₂PO₄.

**Nucleotide-induced Spectral Changes**—Slow kinetic measurements were performed using a Cary 1 spectrophotometer (Varian). Reaction mixtures containing HKM buffer and 40 μM Hsc66 were incubated at 23 °C for 5 min prior to initiating the reaction by adding 20 μM ATP or 20 μM ADP/Pi. Spectra were acquired using a data interval of 1 nm and a scan rate of 120 nm/min.

**Nucleotide Association and Dissociation Rates**—Fast kinetic experiments were performed using a stopped-flow UV-visible spectrophotometer (Hi-Tech, UK). Assays were carried out in HKM buffer using 20–40 μM Hsc66 and 0–500 μM ADP or ATP. Experiments were initiated by rapidly mixing equal volumes of protein and nucleotide solutions (100 μl each) that had been pre-equilibrated at 23 °C for 5 min prior to injecting the Michaelis-Menten equation to a plot of Vₕ versus [ATP], was included in all assay buffers. Rate constants requiring a fit of at least squares linear regression analysis of the data and are plotted as the rate observed (Vₕ) versus ATP concentration. The curve shown represents a least squares fit of the data to the Michaelis-Menten equation assuming a Kᵣ = 12.7 μM and a k₉ = 0.00138 s⁻¹.

**Error Analysis**—Data shown in figures are plotted with error bars representing ±1 S.D. for each data point. Kinetic values are reported with ±1 S.D. resulting from the fit to the data, with propagation of error through any subsequent calculations. Rate constants requiring a fit of kₙₑₐ (i.e. kₙ and kₚ for ATP and ADP) are reported as ±1 S.D. from the secondary fit, using all available values of kₙₑₐ.

**RESULTS**

**Steady-state ATPase Activity of Hsc66**—In initial experiments we investigated the steady-state ATPase activity of Hsc66 over a range of ATP concentrations (2–100 μM) at 23 °C (Fig. 1). The value obtained for kₙₑₐ (0.00138 ± 0.00005 s⁻¹) is similar to values reported for DnaK under similar experimental conditions (0.0003–0.0014 s⁻¹; see Refs. 8, 11–13, and 32).

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1 Additional impurities in [α-³²P]ATP may account for the finding in single turnover experiments (Fig. 2) that the fraction of ATP produced is <100%.
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Hsc66, however, exhibits an unusually low affinity for ATP ($K_m = 12.7 \pm 1.8 \mu M$), ~10-fold lower affinity than values reported for DnaK ($K_m \sim 0.019 \mu M$; see Ref. 12).

**The Rate of ATP Hydrolysis**—To examine the rates of individual steps in the Hsc66 ATPase reaction cycle, we first investigated single turnover ATP hydrolysis activity by monitoring the production of [$\alpha$-32P]ADP from [$\alpha$-32P]ATP using methods described previously (12). Experiments were performed using limiting [$\alpha$-32P]ATP and high concentrations of Hsc66 such that most of the nucleotide will be bound rapidly compared with the rate of hydrolysis. Under these conditions, the observed rate for conversion of ATP to ADP is described by Equation 1 (32). This equation can be simplified to Equation 2 (where hyd is hydrolysis and syn is synthesis).

\[
\begin{align*}
[\text{ADP}^+] &= \text{[ADP]_o} \cdot [1 - (k_{\text{hyd}}/k_{\text{hyd} + k_{\text{syn}}})] \cdot e^{-k_{\text{hyd}} t} \\
\text{[ADP]} &= \text{[ADP]_o} \cdot (1 - e^{-k_{\text{cat}} t})
\end{align*}
\] (Eq. 1)

(Eq. 2)

The observed rate for conversion of ATP to ADP is described by a single exponential ($k_{\text{obs}} = 0.00143 \text{ s}^{-1}$) and maximal hydrolysis is 92.8%.

Hydrolysis reactions containing 150 $\mu M$ Hsc66 and 50 $\mu M$ ATP were allowed to proceed for 48 h at 37 °C, and 31P NMR was then used to investigate the distribution of 18O species in inorganic phosphate. Each 18O incorporated into phosphate is expected to cause a ~0.02 ppm change in the signal arising from phosphate (35). At 48 h, peaks corresponding to the $\alpha$, $\beta$, and $\gamma$-phosphates in ATP were no longer detectable, whereas those corresponding to the $\alpha$- and $\beta$-phosphates in ADP were observed indicating hydrolysis had proceeded to completion (data not shown). The resonance for phosphate produced in reactions lacking enriched water appeared as a singlet with a resonance at 2.159 ppm (Fig. 3A). Resonances for reactions performed in the presence of 50% H$_2^{16}$O appeared as a doublet with peaks at 2.159 and 2.141 ppm consistent with the presence of both P$_{16O}$ and P$_{16O}$P$_{18O}$ (Fig. 3B). Because 31P NMR is capable of detecting phosphate concentrations >1 mM under the experimental conditions used, we conclude that <2% of the phosphate molecules produced from ATP hydrolysis have two or more 18O atoms incorporated into them. Based on these results, we estimate that the rate of ATP synthesis ($k_{\text{syn}} \leq 3 \times 10^{-5} \text{ s}^{-1}$) is at least 50-fold slower than the rate of ATP hydrolysis and thus is unlikely to occur in the time course of physiological processes.

**ATP Binding Kinetics**—For DnaK and some other hsp70 proteins, nucleotide-induced fluorescence changes have been used to determine the kinetics of hsp70 and nucleotide interactions (11, 13, 15, 36–39). We investigated whether nucleotides affect the spectral properties of Hsc66 and found that ATP binding induces a red shift in the near-UV absorption spectrum of Hsc66. The difference spectrum induced immediately following ATP addition is shown in Fig. 4. This ATP-induced spectral change is time-dependent and decays to that resembling the spectrum observed upon mixing Hsc66 and ADP or Hsc66 and ADP plus P. The rate of disappearance of the ATP-induced spectral change under these single turnover conditions can be described by a single exponential (inset, Fig. 4). The observed rate of decay ($k_{\text{decay}} = 0.00126 \pm 0.00003 \text{ s}^{-1}$) is similar to the rate observed for ATP hydrolysis ($k_{\text{hyd}} = 0.00143 \text{ s}^{-1}$) suggest-
ing that the state giving rise to this difference absorbance spectrum requires bound ATP.

The large magnitude of the difference spectra induced upon mixing Hsc66 with ATP (Δε280 = 2 × 10^4 M⁻¹ cm⁻¹) or ADP/ P_i, (Δε280 = 500 M⁻¹ cm⁻¹) provides a useful probe for investigating Hsc66 and nucleotide binding kinetics using stopped-flow methods. Fig. 5A shows a representative plot of the rate of the Δε280 formation upon mixing 75 μM ATP and 20 μM Hsc66. Residuals for single and double exponential fits to this data, Equations 3 and 4, indicate that the formation of the ATP-induced difference spectrum is biphasic, and the curve shown represents a double exponential fit to the data. The first rapid exponential comprises ~70% of the Δε280 and displays a rate (kfast) = 4.9 ± 0.2 s⁻¹; the slower exponential comprises ~30% of the Δε280 signal and displays a rate (kslow) = 0.096 ± 2 × 10⁻¹⁻¹ s⁻¹.

\[ A_t = \Delta A \cdot e^{-k_{fast} t} + C \] (Eq. 3)

\[ A_t = \Delta A_{fast} \cdot e^{-k_{fast} t} + \Delta A_{slow} \cdot e^{-k_{slow} t} + C \] (Eq. 4)

To investigate whether the absorbance changes observed are the result of a bimolecular binding reaction or might represent unimolecular changes in the conformation of the Hsc66-ATP complex following binding, we carried out measurements over a range of ATP concentrations (25–150 μM) with Hsc66 fixed at 20 μM. Fig. 5B and C, shows that kfast, but not kslow, is affected by ATP concentration. The value of kfast increases with ATP concentration in a linear fashion indicating that this rate constant describes an absorbance change associated with the binding of ATP and Hsc66. Because kfast represents the observed rate constant for a reversible binding reaction, the plot of kfast versus [ATP] in Fig. 5B has a slope equal to K_{ATP} (4.2 ± 0.4 × 10⁴ M⁻¹ s⁻¹) and a y intercept equal to k_{ATP} (1.1 ± 0.3 s⁻¹) (40). By using these values to calculate a K_{ATP} for ATP yields a value (K_{ATP} = k_{ATP}/k_{ATP} = 26 ± 7 μM) which is similar to the K_{ATP} (13 μM) determined in steady-state experiments (Fig. 1). Thus the unusually low affinity of Hsc66 for ATP compared with DnaK (K_{ATP} = 0.001–0.007 μM; Refs. 11, 12) arises primarily from the much higher ATP dissociation rate for Hsc66.

In contrast to k_{fast}, k_{slow} is not affected by ATP concentration and displays an average value of 0.10 ± 0.03 s⁻¹. This suggests k_{slow} represents a unimolecular change that follows ATP binding but precedes hydrolysis (k_{hyd} = 0.00143 s⁻¹). We propose that k_{slow} represents a conformational change (k_{conf}) corresponding to the rate of conversion of Hsc66 from the R state (high peptide affinity) to the T state (low peptide affinity) similar to that proposed for other hsp70 proteins (11, 13, 38). The rate of this ATP-induced unimolecular transition for Hsc66 is slower than that observed for DnaK under similar conditions (k ~ 0.67–1.5 s⁻¹; Refs. 11 and 13).

**ADP Binding Kinetics**—Stopped-flow methods were also used to investigate the kinetics of ADP and Hsc66 binding by monitoring the small increase in absorbance at 280 nm which occurs upon ADP binding. Initial experiments were performed in the absence of inorganic phosphate, and Fig. 6A shows a representative plot of the rate of the Δε280 formation upon mixing 100 μM ADP and 40 μM Hsc66. The data fit to a single exponential (k_{obs} = 129 ± 9 s⁻¹), and the residuals for this model (inset, Fig. 6A) indicate the data are monophasic. Fig. 6B shows that the rate of Δε280 formation is a linear function of ADP concentration and arises from Hsc66 and ADP binding. The slope of a linear fit (k_{obs}) to this data yields k_{ADP} = 7.0 ± 0.9 × 10⁵ M⁻¹ s⁻¹, and the y intercept yields k_{ADP} = 60 ± 9 s⁻¹. The rate of ADP binding to Hsc66 is similar to that previously reported for DnaK (k_{ADP} = 10⁵ M⁻¹ s⁻¹; Ref. 12). The rate of ADP dissociation for Hsc66, however, is extremely fast compared with that previously reported for DnaK (k_{ADP} < 0.006–0.035 s⁻¹; Refs. 11–13), and this results in a much lower ADP affinity for Hsc66 (K_{ADP} = k_{ADP}/k_{ADP} = 87 ± 17 μM) than for DnaK (K_{ADP} = 0.025–0.13 μM; Refs. 11 and 12).

Previous studies on DnaK showed that the presence of inorganic phosphate can slow the rate of ADP release to a value that approaches the rate of ATP hydrolysis (12, 13). As a result of this, the T state (low peptide affinity) and R state (high peptide affinity) of DnaK exhibit similar lifetimes in the absence of cochaperones and peptide substrates. To determine if inorganic phosphate affects Hsc66 and ADP binding kinetics, we investigated the effects of physiological levels of P_i (1–10 mM; Refs. 41–43) on the rate of formation of the ADP-induced Δε280.

**Calorimetric Measurements of ADP and P_i Binding**—Isothermal titration calorimetry (ITC) methods were used to obtain independent measurements of the affinity of Hsc66 for ADP and P_i. Fig. 7A shows a profile for the binding of ADP to Hsc66. The area under each peak represents the heat of each ADP injection, a result of both the heat of binding and the heat of ADP dilution. The integrated heats due to Hsc66 and ADP binding were corrected for the heat of ADP dilution and divided by the moles of ADP injected, and the resulting values (Q_{int}) are

- For ATP (4.2 ± 0.4 × 10⁴ M⁻¹ s⁻¹) and a y intercept equal to k_{ATP} (1.1 ± 0.3 s⁻¹) (40).
- Using these values to calculate K_{ATP} for ATP yields a value (K_{ATP} = k_{ATP}/k_{ATP} = 26 ± 7 μM) which is similar to the K_{ATP} (13 μM) determined in steady-state experiments (Fig. 1).
- Thus the unusually low affinity of Hsc66 for ATP compared with DnaK (K_{ATP} = 0.001–0.007 μM; Refs. 11, 12) arises primarily from the much higher ATP dissociation rate for Hsc66.
- In contrast to k_{fast}, k_{slow} is not affected by ATP concentration and displays an average value of 0.10 ± 0.03 s⁻¹. This suggests k_{slow} represents a unimolecular change that follows ATP binding but precedes hydrolysis (k_{hyd} = 0.00143 s⁻¹). We propose that k_{slow} represents a conformational change (k_{conf}) corresponding to the rate of conversion of Hsc66 from the R state (high peptide affinity) to the T state (low peptide affinity) similar to that proposed for other hsp70 proteins (11, 13, 38).
- The rate of this ATP-induced unimolecular transition for Hsc66 is slower than that observed for DnaK under similar conditions (k ~ 0.67–1.5 s⁻¹; Refs. 11 and 13).
- **ADP Binding Kinetics**—Stopped-flow methods were also used to investigate the kinetics of ADP and Hsc66 binding by monitoring the small increase in absorbance at 280 nm which occurs upon ADP binding. Initial experiments were performed in the absence of inorganic phosphate, and Fig. 6A shows a representative plot of the rate of the Δε280 formation upon mixing 100 μM ADP and 40 μM Hsc66. The data fit to a single exponential (k_{obs} = 129 ± 9 s⁻¹), and the residuals for this model (inset, Fig. 6A) indicate the data are monophasic. Fig. 6B shows that the rate of Δε280 formation is a linear function of ADP concentration and arises from Hsc66 and ADP binding. The slope of a linear fit (k_{obs}) to this data yields k_{ADP} = 7.0 ± 0.9 × 10⁵ M⁻¹ s⁻¹, and the y intercept yields k_{ADP} = 60 ± 9 s⁻¹. The rate of ADP binding to Hsc66 is similar to that previously reported for DnaK (k_{ADP} = 10⁵ M⁻¹ s⁻¹; Ref. 12). The rate of ADP dissociation for Hsc66, however, is extremely fast compared with that previously reported for DnaK (k_{ADP} < 0.006–0.035 s⁻¹; Refs. 11–13), and this results in a much lower ADP affinity for Hsc66 (K_{ADP} = k_{ADP}/k_{ADP} = 87 ± 17 μM) than for DnaK (K_{ADP} = 0.025–0.13 μM; Refs. 11 and 12).

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plotted versus the molar ratio of ADP to Hsc66 (Fig. 7B). The resulting binding isotherm was fitted by nonlinear least square simulation using the program ORIGIN (27). Analysis yielded 0.96 ± 0.03 ADP-binding sites, $D_H^0 = -5.8 ± 0.3$ kcal/mol, and a $K_D^{ADP}$ (51 ± 4 µM) similar to that calculated from stopped-flow rate measurements ($K_D^{ADP} = 87$ µM). Experiments were also carried out in the presence of 10 mM phosphate, and analysis yielded 1.1 ± 0.2 ADP-binding sites, $D_H^0 = -3.4 ± 0.8$ kcal/mol, and a $K_D^{ADP}$ (248 ± 36 µM) similar to that calculated from stopped-flow measurements (325 µM) performed in the presence of phosphate (data not shown). The ITC results thus provide an independent confirmation of the kinetic results.

Isothermal titration calorimetry was also used to investigate the equilibrium binding constant of inorganic phosphate to Hsc66. The integrated heats due to Hsc66 and Pi binding were corrected for the heat of Pi dilution and divided by the moles of Pi injected, and the resulting values ($Q_{inj}$) are plotted versus the molar ratio of Pi to Hsc66 (Fig. 7C). The curve shown represents a model for a single phosphate-binding site with a $K_D^{Pi}$ (0.73 ± 0.03 mM) and $D_H^0 = -4.05 ± 0.07$ kcal/mol. This equilibrium binding constant for phosphate is within the range of values previously reported for DnaK ($K_D^{Pi}$; 0.45–2.5 mM; Refs. 12 and 32).

**Fig. 5.** Stopped-flow measurements of ATP binding kinetics. A, kinetics of absorbance changes at 280 nm induced by mixing ATP and Hsc66 in HKM buffer to final concentrations of 75 and 20 µM, respectively, at 23 °C. A double exponential, see Equation 4, is fit to the data and yields $k_{fast} = 4.9$ s$^{-1}$ and $k_{slow} = 0.096$ s$^{-1}$. Inset, residuals for single and double exponential fits. B, plot of $k_{fast}$ versus ATP concentration. The line represents a least squares linear fit to the data and yields a slope of $4.2 × 10^4$ M$^{-1}$ s$^{-1}$ ($k_a^{A TP}$) and a y intercept of 1.1 s$^{-1}$ ($k_d^{ATP}$). C, plot of $k_{slow}$ versus ATP concentration.

**Fig. 6.** Stopped-flow measurements of ADP binding kinetics. A, kinetics of absorbance changes at 280 nm induced by mixing ADP and Hsc66 in HKM buffer in the absence of phosphate to final concentrations of 100 and 40 µM, respectively, at 23 °C. A single exponential ($k_{obs} = 129$ s$^{-1}$) is fit to the data. Inset, residuals for a single exponential fit. B, plot of $k_{obs}$ versus ADP concentration in the absence of inorganic phosphate. The line represents a least squares linear fit to the data and yields a slope of $7.0 × 10^5$ M$^{-1}$ s$^{-1}$ ($k_a^{ADP}$) and a y intercept of 60 s$^{-1}$ ($k_d^{ADP}$). C, plot of $k_{obs}$ versus ADP concentration in the presence of 10 mM Pi. The line represents a least squares linear fit to the data and yields a slope of $0.28 × 10^5$ M$^{-1}$ s$^{-1}$ ($k_a^{ADP(Pi)}$) and a y intercept of 9.1 s$^{-1}$ ($k_d^{ADP(Pi)}$).
13). Attempts to improve further the fit of the model to the data by including additional phosphate-binding sites did not significantly affect the $K_d$ or $D_{H0}$ values of the primary site and yielded only weaker binding sites exhibiting very low enthalpic changes ($D_{H0}$, 1 kcal/mol; data not shown).

The Rate of Phosphate Dissociation—The rapid ADP dissociation and ATP binding rates for Hsc66 suggest that ADP/ATP exchange will be fast compared with ATP hydrolysis. It is possible, however, that the rate of phosphate dissociation could limit the rate of nucleotide exchange. To determine the rate of Pi release, we monitored the rate of ATP binding to Hsc66 $\pi$ complexes assuming that Pi must be released in order for ATP to bind. Hsc66 $\pi$ complexes were initially formed by incubating Hsc66 with a concentration of Pi (10 mM) that should result in $\approx 93\%$ of the protein being complexed with Pi based on the $K_d$ determined in ITC experiments (0.73 mM). The Hsc66 $\pi$ complex was rapidly mixed with a concentration of ATP (5 mM) that is predicted to result in a rapid spectral transition ($k_{ATP}^{\pi} = 4.2 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$) compared with the rate of Pi release. Under conditions where the rate of ATP binding ($k_{ATP}$) is fast compared with the rates of Pi release ($k_{d,\pi}$), Pi association ($k_{\pi}^{\pi}$), and ATP dissociation ($k_{d,ATP}^{\pi} = 1.1 \text{ s}^{-1}$), the rate of formation of ATP-induced spectral transitions arising from Hsc66 $\pi$ complexes is approximated by a single exponential, where $k_{obs}$ is only an approximate measure of the minimal rate of Pi dissociation ($k_{d,\pi}^{\pi} \approx k_{obs}$). Fig. 8 shows a plot of $\Delta A_{288}$ with the data fit to a single exponential. The residuals for this model (inset, Fig. 8) indicate the data can be approximated as monophasic over the time course of acquisition. The rate observed ($k_{d,\pi}^{\pi} \approx 12.1 \pm 0.4 \text{ s}^{-1}$) is similar to that observed for ADP release in the presence of inorganic phosphate ($k_{d,ADP}^{\pi}$) = 9.1 s$^{-1}$; Fig. 6C) and is $=10^2$-fold faster than the rate of ATP hydrolysis. This indicates that the exchange of ATP for ADP and Pi will not contribute significantly to the overall rate of ATP hydrolysis under steady-state conditions.

DISCUSSION

We have performed equilibrium binding, steady-state, and pre-steady-state measurements to characterize the Hsc66 ATPase reaction cycle, and the kinetic parameters obtained from these studies are summarized in Table I. Based on these results, we propose a model for the Hsc66 ATPase reaction.
See text for discussion.

Table I
Summary of experimental results for kinetic measurements of Hsc66 catalyzed ATP hydrolysis at 23 °C

| Parameter | Value (unit) |
|-----------|-------------|
| $K_m$ (μM) | 12.7 ± 1.8 |
| $k_{cat}$ (s$^{-1}$) | 0.00138 ± 0.00005 |
| $k_{	ext{hydrolyze}}$ (s$^{-1}$μM$^{-1}$) | 0.00143 ± 0.00002 |
| $k_{synth}$ (s$^{-1}$μM$^{-1}$) | $3 \times 10^{-5}$ |
| $k_{	ext{decay}}$ (s$^{-1}$μM$^{-1}$) | 0.00126 ± 0.00003 |
| $k_{\text{ATP}}$ (m$^{-1}$ s$^{-1}$) | 4.2 ± 0.4 × 10$^4$ |
| $k_{\text{ATP}}$ (s$^{-1}$) | 1.1 ± 0.3 |
| $K_d^T$ calculated (μM) | 26 ± 7 |
| $k_{\text{conf}}$ (s$^{-1}$) | 0.10 ± 0.03 |
| $k_{\text{ATP}}$ (s$^{-1}$) | 7.0 ± 0.9 × 10$^5$ |
| $k_{d}$ (s$^{-1}$) | 60 ± 9 |
| $K_{d}^{\text{ADP}}$ calculated (μM)$^b$ | 87 ± 17 |
| $K_{d}^{\text{ADP}}$ measured (μM)$^b$ | 51 ± 4 |
| $k_{\text{ADP}}^{\text{Pi}}$ (s$^{-1}$μM$^{-1}$) | 0.28 ± 0.04 × 10$^5$ |
| $k_{d}^{\text{ADP}}$ (s$^{-1}$μM$^{-1}$) | 9.1 ± 1.2 |
| $K_{d}^{\text{Pi}}$ calculated (μM)$^b$ | 325 ± 60 |
| $K_{d}^{\text{Pi}}$ measured (μM)$^b$ | 248 ± 36 |
| $K_{d}^{\text{Pi}}$ (mM) | 0.73 ± 0.03 |
| $k_{d}^{\text{Pi}}$ (s$^{-1}$) | $12.1 ± 0.4$ |

$^a$ Values at 37 °C.
$^b$ Equilibrium dissociation constants were calculated by dividing dissociation rate constants by their respective association rate constants.
$^c$ Values at 25 °C.
$^d$ Kinetic parameters were determined in the presence of 10 mM inorganic phosphate.

Scheme 1. Kinetic scheme of the Hsc66 ATPase reaction cycle. See text for discussion.

Hsc66 binds ATP in a two-step process—Hsc66 binds ATP in a two-step process preceding hydrolysis as evidenced by the rapid, biphasic, ATP-induced change in Hsc66 absorbance. The rate constant describing the rapid phase of this spectral change ($k_{\text{fast}}$) is interpreted as arising from formation of an Hsc66/ATP collision complex (Hsc66-ATP) since $k_{\text{fast}}$ increases linearly with ATP concentration. The rate constant describing the second slower phase of the ATP-induced spectral change ($k_{\text{slow}}$) did not vary with ATP concentration and is interpreted as arising from a unimolecular process that follows ATP binding. In previous studies, we found that ATP destabilizes Hsc66-peptide complexes (21) indicating that either ATP binding or the subsequent unimolecular step affects the conformation of the peptide binding domain of Hsc66. We therefore interpret $k_{\text{slow}}$ as representing a slow, conformational change ($k_{\text{conf}}$) converting Hsc66 from a high peptide affinity R state to a low peptide affinity T state (Hsc66-ATP).

ATP Hydrolysis Is Rate-limiting in the Forward Reaction Cycle—ATP hydrolysis occurs with similar rates under both single turnover and steady-state conditions indicating that $k_{\text{hyd}}$ is rate-limiting in the forward reaction cycle. Since this kinetic step occurs with a rate that is >70-fold slower than any other forward step in the reaction cycle, it seems likely that $k_{\text{hyd}}$ will be subject to cochaperone regulation as has been reported for other hsp70 family members (14–17). ATP hydrolysis also appears to be irreversible on the time course of physiological processes since a maximum of one $^{18}$O was incorporated into inorganic phosphate when ATP hydrolysis reactions were performed in H$_2^{18}$O.

Under single turnover conditions, the ATP-induced difference spectrum decays to that resembling the spectrum in the presence of ADP and P$_i$ with a rate ($k_{\text{decay}}$) essentially equal to the rate of ATP hydrolysis. This finding can be explained by a two-step sequential mechanism, in which ATP hydrolysis precedes a conformational change that results in a decay of the ATP-induced difference spectrum. Previous studies showed that Hsc66-peptide complexes are stabilized in the presence of ADP (21). We therefore interpret this hydrolysis-induced spectral change as representing a conformational change in Hsc66 converting it from the T state (low peptide affinity) to the R state (high peptide affinity). Because the observed spectral transition occurs with a rate similar to that observed for ATP hydrolysis, it seems likely that this conformational change occurs with a rate that is faster than that for ATP hydrolysis (>0.0014 s$^{-1}$).

Nucleotide Exchange Is Rapid Compared with ATP Hydrolysis—Analysis of the concentration dependence of ADP-induced absorbance changes indicates that the rate of ADP dissociation is >10$^3$-fold faster than the rate of ATP hydrolysis ($k_{\text{hyd}} = 0.0014$ s$^{-1}$) both in the absence ($k_{d}^{\text{ADP}} = 60$ s$^{-1}$) and in the presence of physiological levels of phosphate ($k_{d}^{\text{ADP}} = 9.1$ s$^{-1}$). In addition, the rate of phosphate dissociation ($k_{d}^{\text{Pi}} = 12$ s$^{-1}$) was found to be >10$^5$-fold faster than the rate of ATP hydrolysis in the absence of ADP. The fast ADP and P$_i$ dissociation rates, taken together with the prediction that ATP binding will be extremely fast in vivo where the ATP concentration is high (~3 mM in logarithmically growing E. coli; Ref. 44), indicates that the rate of ADP/ATP exchange should be >10$^3$-fold faster than the rate of ATP hydrolysis. The rate of ADP/ATP exchange, therefore, should have little effect on the steady-state ATPase rate. Thus, the high peptide affinity R state (Hsc66-ADP) is expected to be short lived compared with the low affinity T state (Hsc66-ATP) in the absence of auxiliary factors and under conditions where ATP levels are high.

Comparison with the ATPase Reaction Cycle of DnaK—Comparison of our findings for Hsc66 and those published for DnaK (11–13) reveals a number of differences in the kinetics of their respective ATPase reaction cycles. Hsc66 exhibits a 103-fold lower affinity for ATP compared with DnaK. The low nucleotide affinity of Hsc66 explains the lack of ATP exchange may not be subject to further stimulation by an auxiliary protein as is the case for DnaK.

For DnaK the rate of ADP release is similar to that of ATP hydrolysis (11–13), whereas for Hsc66 the rate of ADP release is >10$^3$-fold faster than the rate of ATP hydrolysis. Thus, although there will be a close balance between the R and T states for DnaK, the R $\Leftrightarrow$ T equilibrium will be shifted...

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in favor of the low peptide affinity T state for Hsc66. The slow intrinsic nucleotide exchange rate of DnaK may serve to delay peptide release and decrease the concentration of aggregation-prone peptides; the fact that nucleotide exchange can be rate-determining also allows for regulation of DnaK peptide binding by auxiliary proteins. It is interesting, therefore, that although Hsc66 exhibits both in vitro chaperone and ATPase activity at 42 °C (21, 22), Hsc66 cannot suppress the phenotype accompanying dnaK null mutants in which cytosolic proteins aggregate at 42 °C (46). It is possible that Hsc66 fails to substitute for DnaK as a result of differences in their reaction cycle kinetics because Hsc66-ADP-peptide ternary complexes are expected to exhibit shorter lifetimes than DnaK-ADP-peptide complexes under conditions where ATP levels are high. Alternatively, the failure of Hsc66 to substitute for DnaK could arise from differences in the sequence motifs recognized within peptide substrates by Hsc66 and DnaK or from differences in cochaperone regulation.

**Comparison with Eukaryotic Hsc70**—Studies on both wild type hsc70 (28, 47) and the E543K mutant of Hsc70 (Refs. 10, 33, 38, and 47) indicate that nucleotide interactions with hsc70 are quantitatively more similar to those of DnaK than to those of Hsc66. Both native hsc70 and the E543K mutant exhibit affinities for ATP and ADP (10, 28, 33, 38) that are significantly higher than those reported herein for Hsc66. As was the case with DnaK, the differences in affinities result from large differences in nucleotide dissociation rates, with hsc70 and hsc70(E543K) exhibiting rates that are >10- to >102-fold slower than those observed for Hsc66. The slower rates of ADP release for hsc70 and the E543K mutant indicate that their rates of ADP/ATP exchange will be ~10 to 102-fold faster than their rates of ATP hydrolysis (10, 33, 47), whereas for Hsc66, nucleotide exchange will be >103-fold faster than hydrolysis. For hsc70, cochaperones have been identified that stimulate (Bag-1; see Ref. 48) and inhibit (Hip; see Ref. 49) the rate of nucleotide exchange. To date no proteins have been identified that affect the rate of nucleotide exchange for Hsc66, but the rapid dissociation of ADP from Hsc66 raises the possibility that regulation of the reaction cycle could occur by slowing ADP release as with hsc70; this could act to increase the lifetime of the high peptide affinity R state.

**Cochaperone Regulation**—Hsc20 increases the steady-state ATPase activity of Hsc66 (21, 22), but the mechanism of stimulation remains unknown. Similarities between the N-terminal 70 residues of Hsc20 and the N-terminal J-domain of DnaJ suggest that Hsc20 functions as a J-type ATP hydrolysis stimulatory factor (21), and this hypothesis is further supported by the finding herein that ATP hydrolysis is the rate-limiting step in the Hsc66 ATPase reaction cycle. Whereas the maximal stimulation of Hsc66 by Hsc20 is ~4–6-fold under steady-state conditions (21, 22), DnaJ stimulates the rate of ATP hydrolysis of DnaK up to ~103-fold (14–17). Because the rate of ATP hydrolysis by Hss66 is >70-fold slower than any other forward step in the ATPase reaction cycle, this raises the possibility that additional auxiliary factors could also regulate the reaction cycle of Hsc66 by further increasing the rate of ATP hydrolysis. The genes encoding Hsc66 and Hsc20 are localized to a gene cluster (iscSUASCA-fdx) encoding four additional proteins (20), and the products of one or more of these genes may interact with Hsc66 and affect its steady-state ATPase activity.

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