Substrate Binding to the Molecular Chaperone Hsp104 and Its Regulation by Nucleotides*

Benjamin Bösl1, Valerie Grimminger1, and Stefan Walter2

From the Department Chemie, Technische Universität München, 85747 Garching, Germany

The Hsp104 protein from Saccharomyces cerevisiae is a member of the Hsp100/Clp family of molecular chaperones. It mediates the solubilization of aggregated proteins in an ATP-dependent process assisted by the Hsp70/40 system. Although the principal function of Hsp104 is well established, the mechanistic details of this catalyzed disaggregation are poorly understood. In this work, we have investigated the interaction of Hsp104 with reduced, carboxymethylated α-lactalbumin (RCMLα), a permanently unfolded model substrate. Our results demonstrate that the affinity of Hsp104 toward polypeptides is regulated by nucleotides. In the presence of ATP or adenosine-5’-O-(3-thiotriphosphate), the chaperone formed complexes with RCMLα, whereas no binding was observed in the presence of ADP. In particular, the occupation of the N-terminally located nucleotide-binding domain with ATP seems to be crucial for substrate interaction. When ATP binding to this domain was impaired by mutation, Hsp104 lost its ability to interact with RCMLα. Our results also indicate that upon association with a polypeptide, a conformational change occurs within Hsp104 that strongly reduces the dynamics of nucleotide exchange and commits the bound polypeptide to ATP hydrolysis.

Molecular chaperones are important constituents of the cellular protein quality control system (1, 2). In response to severe growth conditions, the synthesis of many chaperones is up-regulated to cope with potentially harmful consequences of cellular stress such as protein unfolding and aggregation. The Hsp100/Clp chaperone family, a subclass of the AAA+3 proteins, is involved in the unfolding and subsequent degradation of misfolded or damaged polypeptides (3, 4) as well as in the resolubilization of protein aggregates (5–7). The high degree of sequence homology suggests that, despite their diverse functions, Hsp100/Clp proteins employ a common, ATP-dependent mechanism for substrate interaction. When ATP binding to this domain was impaired by mutation, Hsp104 lost its ability to interact with RCMLα. Our results also indicate that upon association with a polypeptide, a conformational change occurs within Hsp104 that strongly reduces the dynamics of nucleotide exchange and commits the bound polypeptide to ATP hydrolysis.

1 The abbreviations used are: AAA+, ATPases associated with various cellular activities; ATP-5S, adenosine-5’-O-(3-thiotriphosphate); f-RCMLα, fluorescence-labeled RCMLα; Hsp104Δϕ6, Hsp104 harboring the mutations E285Q/E667Q; Hsp104Δϕ6ΔN1, wild type Hsp104; NBD, nucleotide-binding domain; RCMLα, reduced carboxymethylated α-lactalbumin; SEC, size-exclusion chromatography; HPLC, high pressure liquid chromatography.

2 To whom correspondence should be addressed: Dept. Chemie, Technische Universität München, Lichtenbergstr. 4, 85747 Garching, Germany. Tel: 49-89-289-13191; Fax: 49-89-289-13345; E-mail: stefan.walter@ch.tum.de.

3 This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 594 (to S. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

4 The on-line version of this article (available at www.jbc.org) contains supplemental Figs. 1 and 2.

5 Both authors contributed equally to this work.

6 The abbreviations used are: AAA+, ATPases associated with various cellular activities; ATP-5S, adenosine-5’-O-(3-thiotriphosphate); f-RCMLα, fluorescence-labeled RCMLα; Hsp104Δϕ6, Hsp104 harboring the mutations E285Q/E667Q; Hsp104Δϕ6ΔN1, wild type Hsp104; NBD, nucleotide-binding domain; RCMLα, reduced carboxymethylated α-lactalbumin; SEC, size-exclusion chromatography; HPLC, high pressure liquid chromatography.

8 This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 594 (to S. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

9 The on-line version of this article (available at www.jbc.org) contains supplemental Figs. 1 and 2.

10 Both authors contributed equally to this work.

11 To whom correspondence should be addressed: Dept. Chemie, Technische Universität München, Lichtenbergstr. 4, 85747 Garching, Germany. Tel: 49-89-289-13191; Fax: 49-89-289-13345; E-mail: stefan.walter@ch.tum.de.

12 The abbreviations used are: AAA+, ATPases associated with various cellular activities; ATP-5S, adenosine-5’-O-(3-thiotriphosphate); f-RCMLα, fluorescence-labeled RCMLα; Hsp104Δϕ6, Hsp104 harboring the mutations E285Q/E667Q; Hsp104Δϕ6ΔN1, wild type Hsp104; NBD, nucleotide-binding domain; RCMLα, reduced carboxymethylated α-lactalbumin; SEC, size-exclusion chromatography; HPLC, high pressure liquid chromatography.

13 This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 594 (to S. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

14 The on-line version of this article (available at www.jbc.org) contains supplemental Figs. 1 and 2.

15 Both authors contributed equally to this work.

16 To whom correspondence should be addressed: Dept. Chemie, Technische Universität München, Lichtenbergstr. 4, 85747 Garching, Germany. Tel: 49-89-289-13191; Fax: 49-89-289-13345; E-mail: stefan.walter@ch.tum.de.

17 The abbreviations used are: AAA+, ATPases associated with various cellular activities; ATP-5S, adenosine-5’-O-(3-thiotriphosphate); f-RCMLα, fluorescence-labeled RCMLα; Hsp104Δϕ6, Hsp104 harboring the mutations E285Q/E667Q; Hsp104Δϕ6ΔN1, wild type Hsp104; NBD, nucleotide-binding domain; RCMLα, reduced carboxymethylated α-lactalbumin; SEC, size-exclusion chromatography; HPLC, high pressure liquid chromatography.

18 This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 594 (to S. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

19 The on-line version of this article (available at www.jbc.org) contains supplemental Figs. 1 and 2.

20 Both authors contributed equally to this work.

21 To whom correspondence should be addressed: Dept. Chemie, Technische Universität München, Lichtenbergstr. 4, 85747 Garching, Germany. Tel: 49-89-289-13191; Fax: 49-89-289-13345; E-mail: stefan.walter@ch.tum.de.
on the one side and substrate binding and release on the other side may reflect a general feature of AAA+ ATPase family members.

MATERIALS AND METHODS

Cloning of Hsp104 Variants and Purification—Site-directed mutagenesis of Hsp104 was performed using polymerase chain reaction with the QuikChange™ site-directed mutagenesis kit (Stratagene). A plasmid carrying the Hsp104 gene was used as template for mutagenesis (24). The following mutations were introduced using appropriate mutagenesis primers: 1) K218T (AAG to ACC), 2) E285Q (GAA to CAG), and 3) K620T (AAA to ACC). The Hsp1044TRAP variant was obtained by introducing the mutation E687Q (GAA to CAG) into the gene of the E285Q single mutant. All mutations were verified by DNA sequencing. After overproduction in E. coli strain BL21-Codon-Plus(DE3)-RIL (Stratagene) wild type and mutant forms of Hsp104 were purified as described previously (24). All concentrations of Hsp104 are given with respect to the monomeric form.

Fluorescein Labeling of α-Lactalbumin—10 mg/ml α-lactalbumin (Sigma) were labeled by adding 2.3 mM fluorescein isothiocyanate as described by the manufacturer (Molecular Probes). The protein was purified via Sephadex G25 column (Amersham Biosciences) equilibrated with 0.2 M Tris/HCl, 2 mM EDTA, pH 7.5, concentrated to a final concentration of 2 mM and carboxymethylated (see below).

Preparation of Reduced, Carboxymethylated α-Lactalbumin (RCMLa)—1.3 mM α-lactalbumin was unfolded and reduced in 0.2 M Tris/HCl, 7 mM guanidinium chloride, 20 mM dithiothreitol, 2 mM EDTA, pH 8.7, for 90 min at room temperature. Subsequently, iodoacetate was added to a final concentration of 0.1 M. After 20 min of incubation under dark room conditions, the reaction was quenched with an excess of reduced glutathione in 0.2 M Tris/HCl, pH 7.5. The derivatized protein was purified using a Sephadex G25 column equilibrated with 20 mM sodium phosphate, pH 7.5.

Size Exclusion Chromatography—Samples containing Hsp104, fluorescein-labeled RCMLa (f-RCMLa), and nucleotides at various concentrations were incubated at 30 °C for 5 min in standard buffer (50 mM Hepes/KOH, 150 mM KCl, 10 mM MgCl2, pH 7.5). 80 μl of the sample were separated on a Superdex 200 HR10/30 column (Amersham Biosciences) equilibrated with 50 mM Tris/HCl, 150 mM KCl, 20 mM MgCl2, 5% glycerol (w/v), pH 7.5. The flow rate was 0.5 ml/min. Absorbance of f-RCMLa was monitored at 494 nm.

ATP Hydrolysis—Rates of ATP hydrolysis were determined by incubating 5 μM Hsp104 and 30 μM RCMLa in standard buffer at 30 °C. The reaction was started by adding 2.5 mM ATP in standard buffer containing 5 mM [32P]ATP. Aliquots (5 μl) were taken at 0, 20, 40, 60, and 90 min, and hydrolysis was stopped with 10 μl of 50 mM Tris/HCl, pH 7.5, 100 mM EDTA. To determine ratios of ATP to ADP and released thiophosphate, reaction mixtures were separated on polyethylene terephthalate-cellulose TLC plates using 0.4 M LiCl, 1.5 M formic acid as the mobile phase. Spot intensities were quantified on a phosphor screen with a Typhoon PhosphorImager (Amersham Biosciences).

Refolding of Denatured Luciferase—Firefly luciferase (7 μM) (Promega) was denatured for 30 min at room temperature in 25 mM Hepes/KOH, pH 7.5, 50 mM KCl, 15 mM MgCl2, 5 mM ATP, 10 mM dithioerythritol, 7 mM urea. Refolding was initiated by 125-fold dilution in 25 mM Hepes/KOH, pH 7.5, 50 mM KCl, 15 mM MgCl2, 5 mM ATP, 2 mM dithioerythritol, 240 μM coenzyme A, 0.05 mg/ml bovine serum albumin, 10 mM phosphoenolpyruvate, 0.1 mM luciferin, 50 μg/ml pyruvate kinase as described previously (25). As chaperones, 2.5 μM Hsp104, 1 μM Sis1, and 1 μM Sis1 were present. Refolding kinetics at 25 °C were recorded for 120 min using a luminometer (Tecan GENios, Crailsheim, Germany).

Analytical Ultracentrifugation—Sedimentation velocity experiments were performed in a Beckman Optima XL1 analytical ultracentrifuge equipped with UV-visible and interference optics. Rotation speed for sedimentation was 50,000, 55,000, or 60,000 rpm (Ti-60 rotor). Data scans were recorded continuously throughout the experiment using the absorption optical system at 280 nm or the Rayleigh interference optics. Alumina double sector centerpieces were used that contained buffer and protein solution (5 μM) in separate chambers. Measurements were carried out in standard buffer at 30 °C. In addition, samples contained 1 mM ATP, 1 mM ADP, or 2.5 mM ATP/y. Data analysis was performed using the ultrascan software by B. Demeler (University of Texas Health Science Center, San Antonio, TX).

Dynamic Light Scattering—The diffusion coefficient of Hsp104 was determined by dynamic light scattering. Experiments were performed at a scattering angle of 90° using an AIXIOS-150 apparatus (Triton-Hellas) with a 35-milliwatt diode laser operating at 658 nm. Conditions were 5–20 μM Hsp104, with and without 1 mM ADP in standard buffer at 20 °C. The particle distributions were obtained using Provencher’s regularized Laplace inversion CONTIN algorithm for the autocorrelation function (26).

The diffusion coefficient D and the sedimentation coefficient s20,w were used to calculate the molecular mass (M) of Hsp104 according to the Svedberg equation,

\[ \frac{s_{20,w}}{D} = \frac{M(1 - \nu_p)}{RT} \]  

\[ \text{(Eq. 1)} \]

in which ν is the partial specific volume of Hsp104 (0.737 ml/g) and ρ is the density of water.

RESULTS

Substrate Binding to Hsp104 Is Dependent on the Presence of ATP—Hsp104 was shown to support the refolding of aggregated enzymes such as luciferase in vitro (5), but attempts to obtain stable chaperone–polypeptide complexes have been futile so far. To investigate the

| Table One | Nucleotide binding and hydrolysis properties of Hsp104 WT and Hsp104 TRAP |
|-----------|---------------------------------|
| Kcat (ATP) | 4 μM | ND |
| Kcat (ADP) | 16 μM | 50 μM |
| kcat | <0.05 min⁻¹ | 70 min⁻¹ |

| Table Two | Analytical Ultracentrifugation |
|-----------|--------------------------------|
| M (WT) | 50,000Da | 55,000Da | 60,000Da |
| s20,w (WT) | 5–20 | μM | Hsp104WT, with and without 1 mM ADP in standard buffer | 20 °C |
| Data for the wild type protein were taken from Ref. 24. ND, not determined. |

ND, not determined.
Substrate Binding by Hsp104

![Image](50x458 to 300x552)

**FIGURE 1.** Substrate binding to Hsp104 is strictly dependent on ATP. *A*, Hsp104TRAP (40 μM) and f-RCMLa (4 μM) were incubated for 5 min at 30 °C in the presence of 2 mM ATP (○), 2 mM ADP (■■■), or without nucleotide (■■■■). Samples were analyzed by SEC-HPLC, and the elution profile of f-RCMLa was recorded using the absorbance of the fluorescein label at 494 nm. *B*, binding of f-RCMLa to Hsp104TRAP monitored by fluorescence anisotropy. f-RCMLa (0.1 μM) was incubated with 1 μM Hsp104TRAP in standard buffer at 30 °C in a fluorescence spectrometer. After 120 s, 1 mM concentration of either ATP (○) or ADP (■■) was added, and the change in anisotropy of the fluorescein label at 515 nm was recorded. *C*, binding of f-RCMLa to Hsp104TRAP is only detectable in the presence of the slowly hydrolyzable analogue ATPγS. Hsp104TRAP (1 μM) and f-RCMLa (0.1 μM) were incubated as described for *B*, and binding reactions were started by the addition of 1 mM ATPγS (○) or 1 mM ATP (■■).

**TABLE TWO**

| Nucleotide | Hsp104WT | K218T | K620T | E285Q/E687Q |
|------------|---------|-------|-------|-------------|
| ATP        | ND      | ND    | ND    | 16.3 S      |
| ATPγS      | 16.5 S  | 15.9 S| 15.5 S| 16.0 S      |
| ADP        | 15.5 S  | 16.5 S| ND    | 16.2 S      |

Sedimentation velocity analysis of Hsp104. The sedimentation velocity of Hsp104 was measured in an analytical ultracentrifuge at 30 °C in standard buffer. Experiments were carried out in the presence of 1 mM ATP, 1 mM ADP, or 2.5 mM ATPγS. Sedimentation coefficients of Hsp104 were determined by the method of van Holde and Weischet (44). ND, not determined.

As expected, we were unable to detect binding of f-RCMLa to Hsp104 using size exclusion chromatography, irrespective of whether ATP, ADP, or no nucleotide was present. Only when the slowly hydrolyzable ATP analogue ATPγS was used, a slight decrease in the elution time of f-RCMLa was observed (data not shown) indicating a transient interaction with the chaperone. This suggested that only the ATP state of Hsp104 is capable of polypeptide binding, similar to what has been described for other AAA ATPases including the bacterial Hsp104 homologue ClpB (30, 31). Because Hsp104 is a fast ATPase with a cat of 70 min⁻¹ (20, 24), the lifetime of the ATP state presumably is too short to detect substrate complexes in SEC analysis. We therefore constructed an Hsp104 variant that can still bind ATP (but is unable to hydrolyze it) by replacing two conserved Glu residues in the Walker B motifs of both NBDs with Gln (E285Q/E687Q) (31, 32). The data summarized in **TABLE ONE** demonstrate that this double mutant, Hsp104TRAP, binds ADP with a slightly higher affinity than the wild type protein but displays no ATPase activity. Moreover, the structure of Hsp104 was found to be unaffected by the mutations we introduced, as assessed by far UV CD spectroscopy (see supplemental Fig. 1).

When the trap mutant was incubated with f-RCMLa and ATP, we detected a substrate-specific peak (λ = 494 nm) at the position of the Hsp104 hexamer in SEC analysis, i.e. the formation of stable Hsp104TRAP·ATP·RCMLa complexes (Fig. 1A). Apparently, the inability of the mutant to hydrolyze bound ATP traps it in a state with a high affinity for polypeptides. When ADP or no nucleotide was present during incubation, no complexes were observed, indicating that substrate binding to Hsp104TRAP is strictly ATP-dependent.

**TABLE ONE**

Oligomeric state of Hsp104 in the presence of nucleotides

| Nucleotide | Hsp104WT | K218T | K620T | E285Q/E687Q |
|------------|---------|-------|-------|-------------|
| ATP        | ND      | ND    | ND    | 16.3 S      |
| ATPγS      | 16.5 S  | 15.9 S| 15.5 S| 16.0 S      |
| ADP        | 15.5 S  | 16.5 S| ND    | 16.2 S      |

SEC-HPLC has the disadvantage of being a slow non-equilibrium method, and labile complexes may irreversibly dissociate during the time course of analysis. Thus, we employed fluorescence anisotropy as an alternative probe to monitor binding of f-RCMLa to Hsp104. The anisotropy signal of a fluorophore depends on its size or more precisely on its rotational correlation time τR (33). Hence, free f-RCMLa (15 kDa) should display a smaller fluorescence anisotropy compared with the large Hsp104–f-RCMLa complex (>600 kDa). The anisotropy signal of f-RCMLa did not change when Hsp104TRAP was added without nucleotide (data not shown), supporting our finding that substrate binding is ATP-dependent. Upon addition of ATP fluorescence anisotropy strongly increased, indicating an increase in τR of f-RCMLa because of its binding to the chaperone (Fig. 1B). Again, ADP was not able to trigger complex formation. When Hsp104WT was used instead of the trap mutant, binding of RCMLa was only observed in the presence of ATPγS but not ATP (Fig. 1C), consistent with our SEC-HPLC data.

We also examined whether the different substrate affinities observed in the presence of ADP and ATP are related to changes in the oligomeric state of the chaperone. In both ADP and ATP, analytical ultracentrifugation yielded sedimentation coefficients, s, of ~16 S (Fig. 2 and TABLE TWO), in agreement with data obtained for hexameric ClpB (34). The integral distribution plot (Fig. 2B) shows that Hsp104 sediments as a single species. Nevertheless, an s value of ~16 S appears to be remarkably small for a 612-kDa protein such as hexameric Hsp104. An accurate determination of the molecular weight from sedimentation velocity data also requires knowledge of the translational diffusion coefficient D. Using dynamic light scattering, we obtained a value of D = 2.5×10⁻⁷ cm²/s. In combination with s = 16 S, this yields a molecular mass of 596 kDa, demonstrating that Hsp104 is completely hexameric under the conditions of our experiments. This conclusion is supported by sedi-
mentation equilibrium analysis of Hsp104, in which Hsp104 was found to consist of a single species of 611 kDa (see supplemental Fig. 2).

Thus, hexamer formation alone (as in the presence of ADP) is not sufficient to trigger substrate binding. Rather an ATP-induced conformational change within the hexamer must be responsible.

NBD1 Regulates the Affinity of Hsp104 for Polypeptides—As Hsp104 contains two nucleotide-binding domains, we tried to assess their roles in polypeptide binding. The mutation of two conserved lysine residues in the Walker A motifs of Hsp104, Lys218 and Lys620, strongly reduces the affinity of the affected NBD for nucleotides, similar to what has been found for Thermus thermophilus ClpB (35). Consequently, the respective mutants only bind nucleotides in the wild type-like NBD but not in the mutated NBD. Fig. 3A shows that the K620T variant of Hsp104, in which only NBD1 can be occupied by nucleotide, still forms complexes with f-RCMLa in the presence of ATP·S. The K218T mutation, in contrast, results in a complete loss of polypeptide binding. These findings strongly suggest that polypeptide substrates can only bind to Hsp104 when NBD1 is occupied with ATP or ATP·S.

To confirm the importance of NBD1, we employed a more stringent assay for Hsp104 function, the disaggregation of luciferase. The K218T mutant, which was unable to bind RCMLa in our anisotropy experiments, also failed to recover any luciferase activity (Fig. 3B). The K620T variant exhibited a significant although reduced disaggregation activity when compared with Hsp104WT. This demonstrates that whereas a functional NBD2 may be dispensable for substrate interaction, it is necessary for efficient disaggregation. The inability of the NBD1 mutant to resolubilize protein aggregates is readily explained by our finding that substrate interaction is crucially dependent on ATP binding to NBD1.

Release of Bound Polypeptide Is Triggered by ATP Hydrolysis—After having determined the requirements for substrate binding to Hsp104, we next investigated the mode of substrate release. In principle, polypeptides could either dissociate from Hsp104 before ATP hydrolysis occurs or remain associated during the hydrolysis step. Likely, only the second pathway is productive, as current models of disaggregation suggest that the energy provided by ATP hydrolysis is used to induce a conformational change in the bound polypeptide (10, 36).

To determine the fate of the bound substrate, ternary complexes between Hsp104 and f-RCMLa were formed as described above. Once fluorescence anisotropy had reached a plateau indicating steady state, we chased the complexes with either (i) a 150-fold excess of unlabeled RCMLa over f-RCMLa or with (ii) a 40-fold excess of ADP over ATP. In both cases, f-RCMLa should be released irreversibly, either because (i) Hsp104 will preferentially reassociate with unlabeled RCMLa or because (ii) excess ADP prevents the binding of ATP necessary to maintain the high affinity state of the chaperone. When experiments were carried out with hydrolytically inactive Hsp104TRAP, the anisotropy signal did not change after the addition of unlabeled RCMLa, irrespective of whether complexes had been formed with ATP or ATP·S. Apparently, the dissociation rate of f-RCMLa is very small and prevents the exchange of bound f-RCMLa with free, unlabeled substrate (Fig. 4A). A very similar result was obtained when the chase was carried out with excess ADP (Fig. 4B).

In contrast to the trap mutant, Hsp104WT·ATP·S was able to release bound f-RCMLa after addition of either unlabeled RCMLa or ADP, as indicated by the decrease in the anisotropy signal (Fig. 4, filled squares). Intriguingly, the rate constant of dissociation (≈0.12 min⁻¹) was the same for both types of chase experiments suggesting that the same molecular step triggers release. Furthermore, our observation that only hydrolytically active Hsp104 can exchange the bound substrate suggests that polypeptide release is tightly coupled with ATP hydrolysis and the concomitant transition of the chaperone into its low affinity ATP state. Under the conditions of our anisotropy experiments, Hsp104WT hydrolyzes [35S]ATP·S ~30 times more slowly (kapp = 0.9 min⁻¹) than ATP. This rate is ~6 times higher than the rate of substrate release, suggesting that multiple hydrolysis events may be required.

One could argue that the release of f-RCMLa from Hsp104WT·ATP·S simply reflects that these ternary complexes are more dynamic than...
Substrate Binding by Hsp104

FIGURE 5. The bound polypeptide strongly reduces the dynamics of nucleotide exchange. A, 5 μm trap mutant was incubated at 30 °C with 0.5 μm ATP to induce the high affinity state of the chaperone. Subsequently, binding of f-RCMLa (0.2 μm) was monitored by fluorescence (fluor.) anisotropy (V). After 4 min, nucleotide exchange was initiated by addition of 20 μm ADP, and substrate release was recorded by following the decrease in anisotropy (•). In a second experiment, the Hsp104TRAPE ATP complex was first incubated for 30 s with 20 μm ADP before f-RCMLa was added (C). If, the same experiment as in A using Hsp104WT and 0.5 μm ATPγS, i.e. under steady-state conditions. C, experimental flow-chart for the order-of-addition experiments. The symbols correspond to those used in A and B.

FIGURE 6. Model for polypeptide binding to Hsp104. Hsp104 rapidly cycles between an ADP state with low affinity toward polypeptides (top right) and an ATP state with high affinity toward polypeptides (bottom right). The cycling time, as judged by the kcat for ATP hydrolysis under steady-state conditions, is in the range of 1 s, and nucleotide exchange should be at least as fast. When a polypeptide substrate is present, it specifically binds to the high affinity form of the chaperone generating the ternary Hsp104-ATP-substrate complex (left). In the case of Hsp104WT, this reaction appears to be quite inefficient, presumably because ATP hydrolysis competes with binding. Our data suggest that substrate binding induces a conformational change in Hsp104 that results in markedly reduced exchange dynamics for both substrate and nucleotide. As a consequence, the ternary complex is committed to hydrolysis. This lock-in mechanism ensure that the energy provided by ATP hydrolysis can be efficiently used for substrate processing.

Hsp104WT, ATP complexes and is not related to hydrolysis of the bound nucleotide. Because the rate of ATPγS hydrolysis is significantly larger than the rate of substrate dissociation, ATPγS becomes hydrolyzed before the polypeptide is released.

Polypeptide Binding to Hsp104-ATP Reduces the Dynamics of Nucleotide Exchange—The observation that ADP is not able to trigger the dissociation of f-RCMLa from ternary Hsp104TRAPE-ATP complexes (Fig. 4B) is remarkable and implies that the exchange of nucleotide in NBD1 must be very slow. Otherwise, ADP should displace ATP and induce the low affinity state of Hsp104. To investigate whether slow nucleotide exchange is a direct consequence of the bound polypeptide, we carried out order-of-addition experiments (see flow chart in Fig. 5).

First, Hsp104TRAPE was incubated with ATP for 10 min to generate the high affinity state. When f-RCMLa was added at this stage (Fig. 5C, left branch of the chart), we observed a large increase in fluorescence anisotropy because of the binding of the labeled polypeptide. As described above, even a 40-fold excess of ADP was not able to cause a significant release of f-RCMLa. This suggests that the ATP in the ternary complex can exchange only very slowly with free ADP. However, when Hsp104TRAPE-ATP was allowed to undergo 30 s of nucleotide exchange with a 40-fold excess of ADP before f-RCMLa was added (Fig. 5C, right branch of the chart), fluorescence anisotropy remained at low levels. Apparently, this short time of exchange was sufficient to convert virtually all Hsp104TRAPE molecules into their low affinity ADP form. Accordingly, replacement of ATP by ADP in NBD1 must occur very rapidly in the absence of a bound polypeptide. To rule out that the slow nucleotide exchange is because of an unusually high polypeptide affinity of the trap mutant, we performed similar order-of-addition experiments with Hsp104WT, in the presence of ATPγS, i.e. under steady-state conditions (Fig. 5B). Again, an ADP chase of 30 s prior to the addition of f-RCMLa was sufficient to block substrate binding. When ADP was added after ternary complexes had been formed, the quantitative release of the bound substrate required more than 1000 s. As described above, this low dissociation rate presumably reflects ATPγS hydrolysis rather than exchange of ATPγS against ADP.

DISCUSSION

Substrate Binding to Hsp104 Is ATP-dependent—A central feature of molecular chaperones is the controlled binding and release of their client proteins (1). In the case of ATP-dependent chaperones, such as GroEL (Hsp60) or DnaK (Hsp70), this is often achieved by conformational changes induced by either nucleotide binding or hydrolysis (37, 38). Clearly, substrate binding to the chaperone Hsp104 is also controlled by nucleotides. In the presence of ATP, Hsp104 adopts a conformation with high affinity for unfolded polypeptides, whereas binding is weak in the presence of ADP or in the absence of nucleotide (cf. model in Fig. 6). This nucleotide-triggered affinity switch seems to be conserved among Hsp100/Cip chaperones, as very similar findings have been reported for ClpA, ClpB, ClpX, and a number of other AAA proteins such as N-ethylmaleimide-sensitive factor (30–32, 39, 40).

Strikingly, binding of RCMLa to Hsp104WT was only detected in the presence of ATPγS but not ATP. Because we used fluorescence anisotropy to monitor complex formation, the transient nature of substrate binding due to ongoing ATP hydrolysis cannot account for this result. Rather, it appears that under conditions of ATP hydrolysis, the steady-state concentration of the ternary complex is below the detection limit. There are two possible explanations: (i) If ATP hydrolysis is much faster than nucleotide exchange, the low affinity ATP state of Hsp104 will predominate under steady-state conditions. This view is corroborated
by the unusually high $K_m$ value of Hsp104 for ATP, which was determined to be in the range of 5–10 mM (24, 41). (ii) ATP hydrolysis may be significantly faster than polypeptide binding, and thus the chaperone will switch back to the low affinity ADP state before RCMLa can bind (Fig. 6). These explanations are not mutually exclusive, and in both cases, a decrease in the hydrolysis rate, either by replacing ATP with ATPγS or by replacing Hsp104K218T with the trap mutant, will cause an increase in substrate binding as observed in our experiments.

*ATP Hydrolysis Is Required for Substrate Release*—Once the polypeptide substrate is associated with Hsp104, it remains bound until ATP hydrolysis occurs. When hydrolysis was blocked as in the case of the Hsp104K218T trap mutant, no spontaneous release of labeled RCMLa could be observed. From a biological point of view, this commitment of the ternary complex ensures that the substrate remains associated with Hsp104 throughout hydrolysis. Therefore, the energy provided by ATP hydrolysis can be efficiently transferred to the substrate and used for its disaggregation, unfolding, or threading through the central pore. Commitment requires that not only the substrate is bound tightly but also ATP. Otherwise, ATP could be replaced by ADP before hydrolysis occurs, resulting in the non-productive release of the polypeptide because of the transition of chaperone to the low affinity state. Our experiments with Hsp104K218T trap show that once a ternary complex (Hsp104K218T:ATP:RCMLa) has been formed, it cannot be dissociated by the addition of excess ADP, indicating that nucleotide exchange is very slow in this complex. Importantly, these statements only apply to NBDs that must maintain their ATP state to keep the substrate bound. Nucleotide exchange in “non-relevant” NBDs is not detected with our experimental setup.

In the absence of a bound polypeptide, nucleotide exchange in Hsp104 must be at least as fast as the rate of steady-state hydrolysis, *i.e.* in the range of 0.1–1 s$^{-1}$. Indeed, when we chased a high affinity Hsp104K218T:ATP complex with an excess of ADP, nucleotide exchange and the concomitant conversion to the low affinity ADP form were complete within seconds. These differences in nucleotide exchange kinetics suggest that nucleotide binding induces a conformational change in Hsp104, which prevents the dissociation of ATP from “relevant” NBDs. This altered conformation may also be the reason why RCMLa and other substrates stimulate the ATP hydrolysis of Hsp104 and ClpB (29).

**NBD1 Is the Primary Regulator for RCMLa Binding to Hsp104**—Like all class I Hsp100/Clp chaperones, Hsp104 possesses two nucleotide-binding domains, both of which could be involved in the control of polypeptide binding. Our results provide evidence that NBD1 serves as the primary regulator. First, a mutant that does not bind nucleotide in NBD1 was also unable to associate with RCMLa, whereas the corresponding mutation in NBD2 did not interfere with substrate binding. The importance of NBD1 is further supported by the inefficient binding of RCMLa to Hsp104K218T:ATP. In our model, this is because ATP hydrolysis in NBD1 is significantly faster than either nucleotide exchange and/or polypeptide binding (see above). Because previous studies (20, 21) have shown that NBD2 is a very slow ATPase under steady-state conditions, this points to NBD1 as the domain responsible for substrate affinity.

Hexamer formation was shown to be essential for Hsp104 chaperone function. A potential concern therefore is that the inability of the K218T mutant to bind polypeptide may be caused by an oligomerization defect. Two points strongly argue against this. (i) Hexamerization alone is not sufficient for polypeptide binding. Hsp104 is hexameric in the presence of ADP and even in the absence of nucleotides at the protein concentrations we used (see TABLE TWO). Under none of these conditions was substrate binding observed. (ii) Lindquist and co-workers (16) demonstrated previously that the K218T mutant displays normal oligomerization behavior, whereas the K620T mutant had an oligomerization defect, which is apparent only at low protein concentration. With respect to polypeptide binding, the effects of the mutations are reversed. The K218T mutant shows a severe defect, whereas the K620T mutant behaves similarly to the wild type protein.

The exact location of the polypeptide binding site on Hsp104/ClpB is not known, although several attempts have been made to address this issue. (i) Cashikar et al. (29) showed that poly-L-Lys binds to the C-terminal domain of Hsp104. This binding site, however, appears to be different from the one investigated in our study, as no dependence on ATP has been reported and a truncated version of Hsp104 lacking NBD1 (and the linker domain, see below) was still able to bind poly-L-Lys. (ii) Based on their crystal structure of *T. thermophilus* ClpB, Tsai and co-workers (10) suggested that the middle/linker domain of the chaperone recognizes aggregated proteins and prions them in a crowbar-like mechanism. Because the linker is inserted in the C-terminal portion of NBD1, a change in the nucleotide status of NBD1 may alter the polypeptide affinity of the linker, consistent with our data. (iii) Recently, Mogk and colleagues (42) showed for ClpB from *E. coli* that peptide substrates can be cross-linked in an ATP-dependent fashion to aromatic residues of NBD1 facing the central pore. Assuming similar roles of NBD1 in both proteins, this would provide a direct structural link between nucleotide binding and polypeptide affinity. The importance of the N-terminal region for substrate binding is also evident from a recent study on ClpB. Mutants, in which the N-terminal domain preceding NBD1 was deleted, showed a marked decrease in affinity toward aggregated polypeptides (43).

**Acknowledgments**—We thank M. Marcinowksi for excellent practical assistance and Nikolaus Neumaier for DLS measurements. We also thank Johannes Buchner and Art Horwich for helpful discussions.

**REFERENCES**

1. Walter, S., and Buchner, J. (2002) *Angew. Chem. Int. Ed. Engl.* 41, 1098–1113
2. Hartl, F. U., and Hayer-Hartl, M. (2002) *Science* 295, 1852–1858
3. Katayama, Y., Gottesman, S., Pumphrey, J., Rudikoff, S., Clark, W. P., and Maurizi, M. R. (1988) *J. Biol. Chem.* 263, 15226–15236
4. Rohrslew, R., Cous, O., Huang, C. F., Moerschell, R. P., You, S. J., Seol, J. H., Chung, C. H., and Goldberg, A. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 5808–5813
5. Glover, J. R., and Lindquist, S. (1998) *Cell* 94, 73–82
6. Goloubinoff, P., Mogk, A., Zwi, A. P., Tomoyasu, T., and Bukau, B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 13732–13737
7. Motohashi, K., Watanabe, Y., Yohda, M., and Yoshida, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 7184–7189
8. Dougan, D. A., Mogk, A., Zeth, K., Turgay, K., and Bukau, B. (2002) *FEBS Lett.* 529, 6–10
9. Zwi A. P., and Goloubinoff, P. (2001) *J. Struct. Biol.* 135, 84–93
10. Lee, S., Sowa, M. E., Watanabe, Y. H., Sigler, P. B., Chiu, W., Yoshida, M., and Tsai, F. T. (2003) *Cell* 115, 229–240
11. Maurizi, M. R., and Xid, D. (2004) *Structure* (Camb.) 12, 175–183
12. Hoskins, J. R., Pak, M., Maurizi, M. R., and Wickner, S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 12135–12140
13. Weber-Ban, E. U., Reid, B. G., Miranker, A. D., and Horwich, A. L. (1999) *Nature* 401, 90–93
14. Lunn, R., Tkach, J. M., Vierling, E., and Glover, J. R. (2004) *J. Biol. Chem.* 279, 29139–29146
15. Weibezehn, J., Tesarz, P., Schlieker, C., Zahn, R., Maglica, Z., Lee, S., Zentgraf, H., Weber-Ban, E. U., Dougan, D. A., Tsai, F. T., Mogk, A., and Bukau, B. (2004) *Cell* 119, 653–665
16. Schirm, E. C., Glover, J. R., Singer, M. A., and Lindquist, S. (1996) *Trends Biochem. Sci.* 21, 289–296
17. Parsell, D. A., Kowal, A. S., Singer, M. A., and Lindquist, S. (1994) *Nature* 372, 475–478
18. Schlie, S., Groemping, Y., Herde, P., Seidel, R., and Reinstein, J. (2001) *J. Mol. Biol.*
Substrate Binding by Hsp104

306, 889–899
19. Mogk, A., Schlieker, C., Strub, C., Rist, W., Weibezahn, J., and Bukau, B. (2003) J. Biol. Chem. 278, 17615–17624
20. Schirmer, E. C., Ware, D. M., Queitsch, C., Kowal, A. S., and Lindquist, S. L. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 914–919
21. Hattendorf, D. A., and Lindquist, S. L. (2002) EMBO J. 21, 12–21
22. Barnett, M. E., and Zolkiewski, M. (2002) Biochemistry 41, 11277–11283
23. Watanabe, Y. H., Takano, M., and Yoshida, M. (2005) J. Biol. Chem. 280, 24562–24567
24. Grimminger, V., Richter, K., Imhof, A., Buchner, J., and Walter, S. (2004) J. Biol. Chem. 279, 7378–7383
25. Beinker, P., Schlee, S., Groemping, Y., Seidel, R., and Reinstein, J. (2002) J. Biol. Chem. 277, 47160–47166
26. Provencher, S. W. (1982) Comput. Phys. Commun. 27, 229–242
27. Hayer-Hartl, M. K., Ewbank, J. J., Creighton, T. E., and Hartl, F. U. (1994) EMBO J. 13, 3192–3202
28. Scholz, C., Stoller, G., Zarrnt, T., Fischer, G., and Schmid, F. X. (1997) EMBO J. 16, 54–58
29. Cashikar, A. G., Schirmer, E. C., Hattendorf, D. A., Glover, J. R., Ramakrishnan, M. S., Ware, D. M., and Lindquist, S. L. (2002) Mol. Cell 9, 751–760
30. Pak, M., Hoskins, J. R., Singh, S. K., Maurizi, M. R., and Wickner, S. (1999) J. Biol. Chem. 274, 19316–19322
31. Weibezahn, J., Schlieker, C., Bukau, B., and Mogk, A. (2003) J. Biol. Chem. 278, 32608–32617
32. Vale, R. D. (2000) J. Cell Biol. 150, F13–F19
33. Lakowicz, J. R. (ed) (1999) Principles of Fluorescence Spectroscopy, 2nd Ed., pp. 291–346, Kluwer Academic/Plenum Publishers, New York
34. Liu, Z., Tek, V., Akoev, V., and Zolkiewski, M. (2002) J. Mol. Biol. 321, 111–120
35. Watanabe, Y. H., Motohashi, K., and Yoshida, M. (2002) J. Biol. Chem. 277, 5804–5809
36. Kenniston, J. A., Baker, T. A., Fernandez, J. M., and Sauer, R. T. (2003) Cell 114, 511–520
37. Staniforth, R. A., Burston, S. G., Atkinson, T., and Clarke, A. R. (1994) Biochem J. 300, 651–658
38. Schmid, D., Baici, A., Gehring, H., and Christen, P. (1994) Science 263, 971–973
39. Whiteheart, S. W., Rossnagel, K., Buhrow, S. A., Brunner, M., Jaenicke, R., and Rothman, J. E. (1994) J. Cell Biol. 126, 945–954
40. Bolon, D. N., Grant, R. A., Baker, T. A., and Sauer, R. T. (2004) Mol. Cell 16, 343–350
41. Schirmer, E. C., Queitsch, C., Kowal, A. S., Parsell, D. A., and Lindquist, S. (1998) J. Biol. Chem. 273, 15546–15552
42. Schlieker, C., Weibezahn, J., Patzelt, H., Tessarz, P., Strub, C., Zeth, K., Erbse, A., Schneider-Mergener, J., Chin, J. W., Schulz, P. G., Bukau, B., and Mogk, A. (2004) Nat. Struct. Mol. Biol. 11, 607–615
43. Barnett, M. E., Nagy, M., Kedzierska, S., and Zolkiewski, M. (2005) J. Biol. Chem. 280, 34940–34945
44. van Holde, K. E., and Weischet, W. (1978) Biopolymers 17, 1387–1403 .0