**trans-Acting Arginine Residues in the AAA+ Chaperone ClpB Allosterically Regulate the Activity through Inter- and Intradomain Communication**

Received for publication, September 1, 2014. Published, JBC Papers in Press, September 24, 2014, DOI 10.1074/jbc.M114.608828

**Cathleen Zeymer, Sebastian Fischer, and Jochen Reinstein**

From the Department of Biomolecular Mechanisms, Max Planck Institute for Medical Research, 69120 Heidelberg, Germany

The molecular chaperone ClpB/Hsp104, a member of the AAA+ superfamily (ATPases associated with various cellular activities), rescues proteins from the aggregated state in collaboration with the DnaK/Hsp70 chaperone system. ClpB/Hsp104 forms a hexameric, ring-shaped complex that functions as a tightly regulated, ATP-powered molecular disaggregation machine. Highly conserved and essential arginine residues, often called arginine fingers, are located at the subunit interfaces of the complex, which also harbor the catalytic sites. Several AAA+ proteins, including ClpB/Hsp104, possess a pair of such trans-acting arginines in the N-terminal nucleotide binding domain (NBD1), both of which were shown to be crucial for oligomerization and ATPase activity. Here, we present a mechanistic study elucidating the role of this conserved arginine pair.

**Background:** Two neighboring, trans-acting arginines in the N-terminal AAA+ domain are essential for oligomerization and activity of ClpB/Hsp104.

**Results:** Both arginines couple nucleotide binding to oligomerization and allosterically regulate the ATPase activity.

**Conclusion:** Site-specifically engineered, cross-linked dimers of AAA+ subunits can be utilized to study allosteric regulation.

**Significance:** This study elucidates the mechanistic role of an essential arginine pair conserved in different AAA+ proteins.

The molecular disaggregation machine ClpB/Hsp104 (caseinolytic peptidase B/heat shock protein 104) is crucial for maintaining protein homeostasis because it reactivates aggregated proteins under cellular stress conditions in concert with the DnaK/Hsp70 chaperone system (1–4). Belonging to the superfamily of AAA+ proteins (ATPases associated with various cellular activities), ClpB/Hsp104 functions as a hexameric complex that converts the chemical energy from ATP hydrolysis into mechanical force (5). Protein disaggregation by ClpB/Hsp104 involves the threading of single polypeptide chains out of the aggregate through the central pore of the hexameric ring (6). High-resolution structural information is available for ClpB from *Thermus thermophilus*, showing a domain architecture that consists of a small N-terminal domain and two highly conserved AAA+ domains, also called nucleotide binding domains (NBD1 and NBD2), per monomer (7). There is a long helical insertion into NBD1, named the M-domain, which was recently identified as a major regulatory element as well as the interaction site for the co-chaperone DnaK/Hsp70 (8–13).

The ATPase modules NBD1 and NBD2 are the motors that drive the molecular machine in a cooperative fashion. The catalytic sites, which are located at the interface between two subunits in the hexameric complex, are built up by highly conserved motifs, namely the Walker A and B motifs that are crucial for nucleotide binding and ATP hydrolysis, respectively (14, 15). Furthermore, there are essential arginine residues, often termed arginine fingers, that contribute to the active sites in trans because they are in close proximity to the nucleotide bound to the adjacent subunit. The role of such conserved arginines in AAA+ proteins has been investigated extensively (16–19). However, it is not trivial to distinguish a truly catalytic arginine finger, as initially identified in GTPase-activating proteins (20), from conserved arginines that either stabilize the hexamERIC state or are crucial for allosteric regulation. The complexity is even increased by the fact that several AAA+ proteins, such as ClpB/Hsp104, ClpA, ClpC, and p97/VCP/Cdc48, possess two highly conserved, neighboring arginines in their NBD1 subunit interface. With this study, we aimed at understanding the mechanistic role of this essential, trans-acting pair of arginines in allosteric communication between AAA+ subunits.

We showed previously that NBD1-M and NBD2 of ClpB from *T. thermophilus* can be expressed and purified separately (21), which allowed a detailed and quantitative characterization of both AAA+ motor domains with regard to nucleotide bind-

---

*This work was supported by the Max Planck Society and by a Ph.D. scholarship from the German National Academic Foundation (to C. Z.).

† To whom correspondence should be addressed: Dept. of Biomolecular Mechanisms, Max Planck Institute for Medical Research, Jahnstrasse 29, 69120 Heidelberg, Germany. Tel.: 49-6221-486-502; Fax: 49-6221-486-585; E-mail: jochen.reinstein@mpimf-heidelberg.mpg.de.

2 The abbreviations used are: NBD1, nucleotide binding domain 1; NBD2, nucleotide binding domain 2; DLS, dynamic light scattering; MANT-dADP, 2'-deoxy-3'-O-(N'-methylanthranilyl)adenosine-5'-O-diphosphate; NBD1-M, ClpB construct comprising residues 141–534; SLS, static light scattering.
Allosteric Regulation and the Role of trans-Acting Arginines

ing, oligomerization, and activity (22–25). Here, we used the construct NBD1-M. Inspired by successful work on the mechanism of ClpX, another AAA+ protein (26–28), we applied a combined approach of covalently linking NBD1-M subunits and introducing Walker A/B and arginine finger mutations. Using these fixed and well determined arrangements of wild-type and mutated subunits in a direct neighborhood, it was possible to dissect the mechanisms of allosteric regulation and intersubunit communication in the AAA+ chaperone ClpB/Hsp104.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Protein Expression, and Purification**—The construct ClpB NBD1-M containing amino acids 141–534 of ClpB from *T. thermophilus* as well as the full-length protein ClpB from *T. thermophilus* were described previously (25, 29). Site-directed mutagenesis was performed using QuikChange PCR according to the QuikChange protocol (Agilent Technologies, Santa Clara, CA) and verified by DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany). The mutations P221C, M394C, Q184C, A390C, R322A, R323A, K204Q, and E271Q were introduced as single mutations and/or in various pairwise combinations. Full-length ClpB and the truncated variant NBD1-M were expressed recombinantly in *Escherichia coli* BL21 (DE3) RIL and purified as described previously (25, 29). 5 mM β-mercaptoethanol was added to all buffers for the purification of cysteine-containing variants. Purified proteins were stored in buffer A (50 mM Tris/HCl, pH 7.5, 50 mM KCl, 5 mM MgCl₂, and 2 mM EDTA), including 5 mM β-mercaptoethanol for cysteine-containing variants.

**Intermolecular Disulfide Bond Formation to Generate Covalently Linked ClpB Dimers**—Cysteine residues were introduced to facilitate the formation of intermolecular disulfide bonds in the ClpB NBD1 subunit interface. The design was based on an available planar hexameric model of ClpB (30). Prior to the reaction, the reducing agent β-mercaptoethanol was removed by buffer exchange. The formation of covalently linked dimers of full-length ClpB or NBD1-M variants using the single cysteine mutant pair P221C/M394C was performed in 50 mM Tris/HCl, pH 7.5, 50 mM KCl, 5 mM MgCl₂. The buffer was EDTA-free because 50 mM copper phenanthroline was used as the oxidizing agent. Equimolar amounts of the respective cysteine variants (50 μM each) were used in a 5-nl reaction volume. 2 mM ADP was added to trigger oligomerization of ClpB variants. The mixture was incubated for 1 h at 37 °C. Subsequently, the reaction mixture was applied to a Superdex 200 26/60 size exclusion column equilibrated with buffer A to separate the monomeric units. The purity of the dimer products was evaluated by non-reducing SDS-PAGE. Test reactions were performed to ensure that homodimer formation was negligible. Another pair of cysteines (Q184C/A390C) was also used to form a covalently linked dimer as described above. However, because this cross-linked variant showed severely impaired ATPase activity, it was not considered for further experiments.

**Steady-state ATPase Measurements**—Steady-state ATPase activity was measured in a coupled colorimetric assay at 25 °C using a JASCO V-650 spectrophotometer (JASCO Germany GmbH, Gross-Umstadt, Germany). ClpB NBD1-M variants were incubated at 25 °C in assay buffer (50 mM Tris/HCl, pH 7.5, 100 mM KCl, 2 mM EDTA, 0.4 mM phosphoenolpyruvate, 0.4 mM NADH, 0.1 g/liter BSA, 4 units/ml pyruvate kinase, 6 units/ml lactate dehydrogenase, and 10 mM MgCl₂). Importantly, reducing agents were strictly excluded from the assay buffer to maintain the intermolecular disulfide bonds of covalently linked dimers. The reaction was started by adding ATP (0.01–8 mM). Measurements were performed for protein concentrations of 1–30 μM (with respect to monomeric units). The decreasing absorption at 340 nm was monitored over time, and the maximal slope was used to determine the ATPase turnover rate per monomer (ε(NADH) = 6220 M⁻¹ cm⁻¹). The data were analyzed with the Hill equation (Equation 1) using the program GraphPad Prism version 5.0.

\[
k = \frac{k_{cat} \cdot [ATP]^n}{k_{m} + [ATP]^n}
\]  

(Eq. 1)

**Stopped Flow Experiments (Determination of MANT-dADP Binding Parameters)**—Nucleotide binding experiments were performed with a BioLogic SFM-400 stopped flow instrument in single mixing configuration (BioLogic Science Instruments, Claix, France) in buffer A at 25 °C essentially as described previously (25). The fluorescently labeled nucleotide MANT-dADP was purchased from BIOLOG (Bremen, Germany). The excitation wavelength was set to 296 nm, and the fluorescence signal was observed using a 400-nm long pass filter (400FG03-25, LOT Oriel Group). This setup was used to selectively excite protein-bound MANT-dADP via fluorescence resonance energy transfer (FRET) from the initially excited tryptophan residues of the protein. Kinetic traces were recorded as triplicates and averaged. Data analysis was performed using the program GraphPad Prism version 5.0.

Kinetic traces from direct binding experiments (2 μM ClpB NBD1-M mixed 1:1 with 10–50 μM MANT-dADP) were fitted to exponential functions. The extracted rate constants were plotted against the nucleotide concentration. The association rate constants \(k_{on}\) for MANT-dADP binding were obtained from the slope of the resulting linear functions. Kinetic traces from dissociation experiments (2 μM ClpB NBD1-M incubated with 15 μM MANT-dADP and subsequently mixed 1:1 with 5 mM Mg-ADP) were fitted to exponential functions; the extracted rate constants correspond to the dissociation rate constants \(k_{off}\) of MANT-dADP binding. The \(K_D\) was calculated from the ratio \(k_{off}/k_{on}\). Given protein concentrations refer to monomeric units.

**Fluorescence Equilibrium Titrations (Determination of ADP/ATP Binding Parameters)**—Fluorescence titrations were performed at 25 °C in buffer A using a Jasco FP-8500 fluorescence spectrometer (Jasco Germany GmbH) as described previously (25). The excitation wavelength was set to 296 nm to facilitate selective excitation of protein-bound MANT-dADP via FRET from nearby tryptophan residues. The MANT fluorescence signal was monitored at 441 nm. Direct titrations of ClpB NBD1-M variants (at 2 or 20 μM) with MANT-dADP (2–50 μM) were used to determine the binding affinity of MANT-dADP, which was subsequently applied as the refer-
ClpB. 0.2

P221C/M394C affects the chaperone activity of full-length

test whether disulfide cross-linking using the cysteine pair

KD

ing

monitored and normalized against a positive control contain-

land). The average rate of absorption increase at 405 nm was

spectrophotometer (Varioskan, Thermo Electron, Vantaa, Fin-

cosidase activity was measured at 40 °C using a microplate

ner. Indicated that both conserved arginines are crucial for ATP

fold reduced ATPase activity compared with the wild type, indi-

ment with previous experiments on full-length ClpB by

Yamasaki et al. (17), the single and double mutants R322A,

R323A, and R322A/R323A, respectively, showed about 1000-

fold reduced ATPase activity compared with the wild type, indica-

ting that both conserved arginines are crucial for ATP

hydrolysis in ClpB NBD1. Next, we tested whether the muta-

tions R322A and R323A influence the nucleotide binding

behavior of ClpB NBD1-M. First, we performed stopped flow

experiments to extract the kinetic nucleotide binding parame-

ters for fluorescently labeled MANT-dADP (Fig. 1B and Table

1). Direct mixing and dissociation experiments showed that

both the single and double mutants were able to bind MANT-

dADP with similar affinities compared with the wild type. This

result is in agreement with previous studies on full-length ClpB

(17). Furthermore, we determined the binding affinities for the

unlabeled nucleotides ADP and ATP by displacement titrations

at low and high protein concentration (2 and 20 μM, respec-

tively) (Fig. 1C and Table 1). Notably, the nucleotide binding

affinities of the single mutants R322A and R323A and the dou-

ble mutant R322A/R323A did not increase at higher protein

concentrations as observed for the wild type, indicating that the

conserved arginines are involved in coupling nucleotide bind-

ing and oligomerization. To further substantiate this hypo-

thesis, we characterized the oligomerization behavior upon nu-

cleotide binding for the single and double mutants using both DLS

and SLS experiments (Fig. 1D and Table 1). In the presence of

ATP, the wild-type protein NBD1-M oligomerizes. With increas-

ing protein concentration, a shift toward trimeric spec-

ies is observed, which correlates tightly with the increase in

ATP hydrolysis rates (Fig. 1E). This together with Hill coeffi-

cients higher than 2.5 indicates that the trimer represents the

smallest hydrolysis-competent unit (Fig. 1F). The nucleotide-

induced oligomerization of NBD1-M is severely impaired by

the R322A and R323A mutations. In contrast to the observed

trimers for the wild-type protein, the molecular masses

obtained for the single and double mutants indicate only a

monomer/dimer equilibrium (Fig. 1D and Table 1). Due to sub-
FIGURE 1. Characterization of ClpB NBD1-M wild type, single mutants R322A and R323A, and double mutant R322A/R323A. A, the ClpB construct NBD1-M comprises amino acids 141–534, which are highlighted in dark gray in the crystal structure of full-length ClpB (Protein Data Bank code 1QVR). The schematic representation for this construct is a gray sphere. ATP is shown in green. The conserved arginines Arg-322 and Arg-323 are shown in blue and pink, respectively. B, stopped flow nucleotide binding experiments. Kinetic fluorescence traces upon direct mixing of NBD1-M R322A/R323A and MANT-dADP are shown (left). The final concentration of protein is 1 μM in all cases. The final MANT-dADP concentration ranges from 5 to 25 μM. Single exponential fits are shown as colored lines. Similar traces were obtained for the single mutants R322A and R323A. The rate constants $k_{\text{on}}$ obtained from fitting the kinetic traces are plotted against the MANT-dADP concentration (right). The association rate constants $k_{\text{on}}$ for MANT-dADP binding were obtained from the slope of the linear functions. The dissociation rate constants $k_{\text{off}}$ can be estimated from the y axis intercepts, but they were determined separately in dissociation experiments, as described under “Experimental Procedures.” C, fluorescence equilibrium titrations. NBD1-M was incubated with MANT-dADP and subsequently titrated with ADP or ATP, respectively. For the ATP titration, phosphoenolpyruvate and pyruvate kinase were present as an ATP-regenerating system. The volume-corrected data were fitted with the cubic equation for competing ligands (31), using $K_D$(MANT-dADP) as an input value. This figure shows the titrations for NBD1-M R322A; similar curves were obtained for all mutants at different protein concentrations. Nucleotide binding parameters extracted from these data are listed in Table 1. D, analytical gel filtration with SLS analysis. Elution profiles of NBD1-M in nucleotide-free buffer (red) and with 2 mM ADP (blue) and with 2 mM ATP (green) present in the running buffer are shown for NBD1-M wild type (top) and NBD1-M R322A/R323A (bottom). The ATP-containing buffer was supplemented with phosphoenolpyruvate and pyruvate kinase as an ATP-regenerating system. Solid line, refractive index signal; dotted line, calculated molecular mass of the eluted species. The actual molecular mass of the NBD1-M monomer is 45 kDa. E, correlation between oligomeric state and ATPase activity. The steady-state ATPase turnover (orange circles) and the molecular mass of oligomeric NBD1-M species (green bars) measured by DLS are plotted in the same diagram for different protein concentrations. The DLS experiments were performed in the presence of 2 mM ATP and phosphoenolpyruvate and pyruvate kinase as an ATP-regenerating system. The increase in ATPase activity correlates with the formation of NBD1-M trimers (F), which represent the smallest ATP hydrolysis-competent unit. a.u., arbitrary units.
**Covalently Linked ClpB Dimers Facilitate Mechanistic Studies on Allosteric Regulation**—With the intention to generate a well defined and fixed ClpB subunit interface, we designed covalently linked NBD1-M dimers with an intermolecular disulfide cross-link (Fig. 2A). Two pairs of cysteines were tested, namely P221C/M394C and Q184C/A390C. The positions were chosen on the basis of an available planar, hexameric ClpB model (30) using the program SSBOND, which suggests positions for cysteine pairs according to the optimal distances and dihedral angles for disulfide bonds (33). Whereas the Q184C/A390C dimer was severely impaired in ATPase activity (100-}

---

**TABLE 1**

Nucleotide binding and oligomerization parameters of ClpB NBD1-M wild type, R322A, R323A, and R322A/R323A

| Mutation                  | Wild-type\(^a\) | R322A   | R323A   | R322A/R323A |
|---------------------------|----------------|---------|---------|-------------|
|                           |                 |         |         |             |
| **Nucleotide binding at [protein] = 1-2 \(\mu M\)** |                 |         |         |             |
| MANT-dADP\(^b\)           |                |         |         |             |
| \(K_\text{on} \ (\mu M^{-1} \text{s}^{-1})\) | 0.34 ± 0.02   | 0.40 ± 0.02 | 0.45 ± 0.01 | 0.45 ± 0.01 |
| \(K_\text{off} \ (\text{s}^{-1})\)         | 3.3 ± 0.03     | 3.3 ± 0.01 | 3.0 ± 0.02 | 3.1 ± 0.01 |
| \(K_D \ (\mu M)\)          | 9.7 ± 0.6      | 8.3 ± 0.4 | 6.7 ± 0.2 | 6.9 ± 0.2 |
| **Unlabeled nucleotides**  |                |         |         |             |
| \(K_D(\text{ADP}) \ (\mu M)\)     | 13.7 ± 1.1     | 10.7 ± 0.6 | 10.2 ± 0.6 | 9.3 ± 0.7  |
| \(K_D(\text{ATP}) \ (\mu M)\)       | 1520 ± 89      | 1670 ± 160 | 1040 ± 120 | 2050 ± 180 |
| **Nucleotide binding at [protein] = 20 \(\mu M\)** |                |         |         |             |
| MANT-dADP\(^c\)           |                |         |         |             |
| \(K_D \ (\mu M)\)          | 5.5 ± 0.5      | 4.9 ± 0.3 | 5.8 ± 0.2 | 7.6 ± 0.7  |
| **Unlabeled nucleotides**  |                |         |         |             |
| \(K_D(\text{ADP}) \ (\mu M)\)     | 6.6 ± 0.3      | 8.7 ± 0.2 | 8.5 ± 0.2 | 11.8 ± 2.3 |
| \(K_D(\text{ATP}) \ (\mu M)\)       | 510 ± 64       | 1720 ± 100 | 1120 ± 65 | 1700 ± 180 |
| **Nucleotide-induced oligomerization measured by DLS at [protein] = 20 \(\mu M\)**\(^d\) |                |         |         |             |
| Nucleotide-free            | 49 kDa         | 47 kDa  | 49 kDa  | 49 kDa      |
| 2 mM ADP                   | 85 kDa         | 62 kDa  | 58 kDa  | 49 kDa      |
| 2 mM ATP\(^e\)            | 134 kDa        | 72 kDa  | 74 kDa  | 69 kDa      |

\(^a\) The characterization of ClpB NBD1-M wild type has been published previously (25). The parameters are given here for comparison.

\(^b\) From stopped flow experiments.

\(^c\) From fluorescence equilibrium titrations.

\(^d\) Measurements were performed in the absence and presence of nucleotide. The molecular mass of monomeric NBD1-M is 45 kDa.

\(^e\) ATP-containing solutions were supplemented with phosphoenolpyruvate and pyruvate kinase as an ATP-regenerating system.
Allosteric Regulation and the Role of trans-Acting Arginines

First, we determined the nucleotide binding parameters of the cross-linked NBD1-M dimer. Stopped flow experiments showed a biphasic fluorescence signal change upon direct mixing with MANT-dADP (Fig. 3A). Both kinetic phases were nucleotide concentration-dependent, indicating the presence of an asymmetric dimer with two unequal nucleotide binding sites. In order to assign the observed phases, we utilized NBD1-M dimers carrying the Walker A mutation K204Q in one of the two subunits (Fig. 2C). These dimers are deficient in nucleotide binding either in the cross-linked or the free active site. Indeed, MANT-dADP binding was monophasic for both variants but with significantly different kinetic parameters \( k_{on} \), \( k_{off} \), and \( K_D \), which allowed an unambiguous assignment (Table 2). Notably, \( K_D \) (MANT-dADP) is significantly lower compared with the unlinked NBD1-M wild type for both active sites of the cross-linked dimer, mainly due to a decrease in the dissociation rate constant \( k_{off} \). This finding again confirms the strong coupling between nucleotide binding and oligomerization in ClpB NBD1. Both association \( (k_{on}) \) and dissociation \( (k_{off}) \) of nucleotide are slower for the cross-linked active site than for the free one. We furthermore determined the binding affinities for unlabeled ADP and ATP by fluorescence displacement titrations and observed 10-fold stronger ADP and 18-fold stronger ATP binding compared with the unlinked NBD1-M (Fig. 3B and Table 2).

Next, we characterized the steady-state ATPase activity of the cross-linked NBD1-M dimer (Fig. 4A and B and Table 3). In agreement with the observed improvement in ATP binding, the dimer showed a significantly lower \( K_m \) and less pronounced dependence of the activity on protein concentration compared with the unlinked NBD1-M. However, the maximum \( V_{max} \) observed for high protein concentrations of unlinked wild-type protein was not reached by the cross-linked dimer. It can be speculated that this is due to the fact that ADP release becomes rate-limiting, considering the measured \( k_{off} \) (MANT-dADP) is lower than 0.1 s\(^{-1}\) for the cross-linked active site. We next generated NBD1-M dimers carrying the Walker B mutation E271Q either in the cross-linked interface or in the free interface. Using these dimers, which are fully nucleotide binding-competent but deficient in...
ATP hydrolysis either in the cross-linked or the free active site, we showed that indeed both of the two different active sites contribute to the overall activity of the dimer (Fig. 4 (A and B) and Table 3). When having the Walker B mutation in the free active site, the remaining activity originating from the cross-linked site is 25% of the cross-linked wild-type, whereas when mutating the cross-linked active site, the free active site is even 30% more active than the wild-type dimer carrying two intact subunits. This somewhat unexpected result emphasizes the importance of allosteric regulation. It seems that a tightly bound ATP molecule in the cross-linked active site activates the neighboring, free active site. To further study this phenomenon, we next measured the ATPase activity of the NBD1-M dimers carrying the Walker A mutation K204Q in one of the two subunits (Fig. 4C) that were already used to assign the nucleotide binding phases. Notably, both variants were inactive (Fig. 4C). Independent of whether the cross-linked or free active site was mutated, it was sufficient to provide a nucleotide binding-deficient nearest neighbor to totally abolish the activity of the intact active site.

Furthermore, it was important to characterize the nucleotide-induced oligomerization of the cross-linked NBD1-M dimer variants. We performed analytical gel filtration runs with SLS analysis (Fig. 3C). The cross-linked wild-type dimer as well as both variants with the Walker B mutation showed pronounced ATP-induced oligomerization. A clear shift toward...
the size of tetramers was observed, indicating that also the cross-linked dimers associate with each other to form higher oligomers in the presence of ATP. In contrast, for both dimer variants carrying the Walker A mutation no nucleotide-induced oligomerization was observed, which is especially interesting for the variant with the cross-linked site being mutated. Here, the absence of nucleotide must be somehow communicated such that the intact and nucleotide binding-competent neighboring subunit cannot associate with another molecule.

In summary, the cross-linked NBD1-M dimers allowed a dissection of allosteric effects in ClpB by site-specifically introducing Walker A and B mutations.

**Allosteric Regulation between ClpB Subunits Is Communicated by Conserved Arginines**—As a next step, it would be desirable to understand the molecular basis of how the observed allosteric regulation is communicated throughout the ClpB oligomer and whether the conserved arginines, Arg-322 and Arg-323, located in the subunit interface are involved in this task. To this end, we studied covalently linked NBD1-M dimer variants carrying either the R322A or R323A mutation in the cross-linked interface (Fig. 2E). This approach was chosen to distinguish a truly regulatory function of the arginines from effects associated with oligomeric stability, the latter of which were assumed to be blanked by covalently fixing the subunit interface. Indeed, SLS measurements confirmed that nucleotide-induced oligomerization of the cross-linked dimers was not impaired by the arginine to alanine mutations (Fig. 3C). In line with this result, both cross-linked dimer variants (R322A and R323A) were nucleotide binding-competent. The biphasic kinetic traces observed upon mixing with the fluorescently labeled MANT-dADP indicated the presence of two intact nucleotide binding sites per dimer. However, slightly higher \( K_D \) values for MANT-dADP were obtained for the mutants compared with the cross-linked wild-type dimer (Fig. 3A and Table 2). In fluorescence displacement titrations, ADP binding was essentially not affected by the arginine to alanine mutations (Fig. 3B and Table 2). This result suggests

---

**TABLE 2**

Nucleotide binding parameters of covalently linked ClpB NBD1-M dimer variants

See Fig. 3 for corresponding experimental data. Stopped flow experiments were performed at [NBD1-M] = 1 \( \mu \)M in the final mixture. Equilibrium titrations were performed at [NBD1-M] = 2 \( \mu \)M, n.d., not determined.

| Mutations                  | Wild-type | R322A | R323A | K204Q Free interface | K204Q Cross-linked interface |
|---------------------------|-----------|-------|-------|-----------------------|-----------------------------|
| **Stopped flow measurements** |           |       |       |                       |                             |
| MANT-dADP binding*        |           |       |       |                       |                             |
| Kinetic behavior          | biphasic  | biphasic | biphasic | monophasic | monophasic                 |
| \( k_{on} \) “free” (s\(^{-1}\)) | 0.57 ± 0.03 | 0.65 ± 0.03 | 0.80 ± 0.06 | -                      | 0.76 ± 0.05                 |
| \( k_{on} \) “cross-linked” (s\(^{-1}\)) | 0.048 ± 0.001 | 0.049 ± 0.001 | 0.065 ± 0.004 | 0.096 ± 0.007 | -                           |
| \( k_{off} \) “free” (s\(^{-1}\)) | 0.38 ± 0.01 | 0.75 ± 0.02 | 1.4 ± 0.06 | -                      | 1.1 ± 0.01                 |
| \( k_{off} \) “cross-linked” (s\(^{-1}\)) | 0.091 ± 0.001 | 0.16 ± 0.001 | 0.31 ± 0.004 | 0.48 ± 0.003 | -                           |
| \( K_D \) “free” (\( \mu \)M) | 0.67 ± 0.04 | 1.2 ± 0.1 | 1.8 ± 0.2 | -                      | 1.4 ± 0.1                 |
| \( K_D \) “cross-linked” (\( \mu \)M) | 1.9 ± 0.1 | 3.3 ± 0.1 | 4.8 ± 0.3 | 5.0 ± 0.4 | -                           |
| **Equilibrium titrations** |           |       |       |                       |                             |
| \( K_D \) (MANT-dADP) (\( \mu \)M) | 3.3 ± 0.3 | 4.3 ± 0.6 | 5.2 ± 0.4 | n.d.                  | n.d.                       |
| Unlabeled nucleotides     |           |       |       |                       |                             |
| \( K_D \) (ADP) (\( \mu \)M) | 1.3 ± 0.1 | 1.2 ± 0.1 | 1.4 ± 0.3 | n.d.                  | n.d.                       |
| \( K_D \) (ATP) (\( \mu \)M) | 96 ± 11 | 438 ± 17 | 255 ± 20 | n.d.                  | n.d.                       |

*The terms “free” and “cross-linked” refer to the two different nucleotide binding sites, one being located in the free (outside) interface and the other one in the cross-linked (inside) interface.
that Arg-322 and Arg-323 interact primarily with the γ-phosphate group of the ATP molecule bound to the neighboring subunit, presumably sensing the nucleotide binding state that way.

Next, we measured the steady-state ATPase activity of the NBD1-M dimers carrying the R322A or R323A mutation in the cross-linked interface (Fig. 4 (C and D) and Table 3). For both variants, the obtained $K_m$ values were significantly increased compared with the cross-linked wild-type dimer, indicating again that the conserved arginines contribute essential interactions that generate cooperativity. Notably, the R323A mutation caused a more severe loss in activity than the R322A mutation (90 and 35% decreased $k_{cat}$ compared with the wild-type dimer, respectively), which might indicate that Arg-322 is mainly involved in stabilizing the subunit interface, which was provided here by the disulfide cross-link. We next asked whether the conserved arginines indeed regulate the activity of a ClpB oligomer by allosteric communication, thus affecting a neighboring catalytic site that they do not directly interact with. To this end, we combined the R322A and R323A mutation with the Walker B mutation E271Q in the cross-linked interface, which allowed studying the direct effect of the arginine mutation on the activity of the catalytic site located in the neighboring, free interface (Fig. 4 (E and F) and Table 3). The cooperativity seemed to be totally blocked (Hill coefficient $n = 1$), and very high $K_m$, low $k_{cat}$, and a strong dependence on protein concentration were observed. These results clearly confirm that the conserved arginines Arg-322 and Arg-323 regulate the highly cooperative ATPase activity of ClpB by allosterically communicating between neighboring subunits, which exceeds the simple mediation of ATP-induced oligomerization.
DISCUSSION

In this study, we investigated the role of the conserved, trans-acting arginines Arg-322 and Arg-323 in allosteric regulation and intersubunit communication in the molecular disaggregation machine ClpB (Fig. 5). Using a simplified system, namely the separate N-terminal ATPase subunit NBD1-M, it was possible to study the interplay between nucleotide binding, oligomerization, and activity in a quantitative manner. We utilized a set of well defined NBD1-M dimers with intermolecular disulfide cross-links and site-specifically introduced Walker A/B mutations to draw conclusions about allosteric effects mediated by the conserved arginine pair.

First, we showed that both arginines are involved in coupling nucleotide binding to oligomerization of ClpB NBD1-M. We identified the NBD1-M trimer as the smallest ATP hydrolysis-competent unit, which is formed upon ATP binding, but only if both arginines are present. The finding that trimer formation is essential is in agreement with previously performed mixing experiments showing that the random incorporation of two mutant ClpB subunits into the hexamer is sufficient to abolish activity (34). A previous study using full-length ClpB came to the conclusion that the trans-acting arginines are not involved in nucleotide binding (17). However, using the cross-linked dimers, we showed that the arginines are indeed crucial for strong and cooperative ATP binding.

The next goal was to obtain a better understanding of allosteric regulation mechanisms implemented in ClpB NBD1-M. A comprehensive mechanistic interpretation of the nucleotide binding, oligomerization, and activity data that we obtained for the different cross-linked dimer variants would greatly benefit from additional structural information about the ClpB subunit interface. The available crystal structure of ClpB from *T. thermophilus* (Protein Data Bank code 1QVR) exhibits a helical arrangement of subunits, thereby displaying a shifted subunit interface (7). The two conserved arginines Arg-322 and Arg-323 are located 5 and 11 Å away from the γ-phosphate of ATP bound to the neighboring subunit, respectively (see Fig. 2A), which may not reflect the active conformation. Several cryo-EM studies on ClpB and its yeast homolog Hsp104 generated models of a planar hexameric ring, which is believed to be the active form (7, 35–37). However, structural details, such as the conformation of the conserved arginines, could not be resolved. When using the hexameric crystal structure of ClpB from *T. thermophilus* (Protein Data Bank code 1QVR) as a template for a planar ClpB model, both conserved arginines are at a 4–6 Å distance from a modeled ATP molecule (38). Still, at this point, there is no reliable knowledge about the exact positioning of the conserved arginine pair in the ClpB subunit interface. Thus, we put great emphasis on control experiments using different Walker A/B mutants to verify our results.

### Table 3

| ATPase activity parameters of covalently linked ClpB NBD1-M dimer variants |
|---------------------------------------------------------------|
| NBD1-M (µM) | kcat (min⁻¹) | Km (µM) | Hill coefficient |
| Wild-type | 5 | 2.32 ± 0.02 | 162 ± 4 | 2.2 ± 0.1 |
| K204Q Free interface | 5 | inactive |
| K204Q Cross-linked interface | 5 | inactive |
| E271Q Free interface | 5 | 0.54 ± 0.01 | 177 ± 4 | 2.4 ± 0.1 |
| E271Q Cross-linked interface | 5 | 3.05 ± 0.05 | 397 ± 11 | 2.8 ± 0.2 |
| R322A | 5 | 1.49 ± 0.05 | 1100 ± 66 | 2.0 ± 0.2 |
| R323A | 5 | 0.25 ± 0.02 | 1390 ± 190 | 1.9 ± 0.4 |
| R322A+E271Q Cross-linked interface | 20 | 0.58 ± 0.03 | 2700 ± 400 | 1b |
| R323A+E271Q Cross-linked interface | 20 | 0.52 ± 0.02 | 2900 ± 300 | 1b |
| Unlinked wild-type a | 5 | 1.4 ± 0.1 | 2200 ± 150 | 2.7 ± 0.3 |

a The characterization of ClpB NBD1-M wild-type has been published previously (25). The parameters are given here for comparison.
b The data showed no sigmoidal shape and were therefore fitted using the Michaelis-Menten equation, which is represented by a simple hyperbolar function (Hill coefficient n = 1).
Allosteric effects related to Walker A/B mutations were studied previously, mainly by using mixing experiments (34, 39–41). The extensive amount of experimental data was complemented recently by a computational study simulating cooperativity in AAA + proteins (42). Franzmann et al. (39) analyzed the allosteric network in Hsp104 and found regulatory circuits in both cis and trans. They concluded that the ATPase activity of a given NBD1 depends on the nucleotide state of the neighboring subunit, which our experiments fully agree with. However, in contrast to this previous study, we observed that the presence of a nucleotide binding-deficient subunit (Walker A mutation) inhibits ATP hydrolysis in the neighboring, intact NBD1-M unit, whereas the presence of a tightly bound ATP in a hydrolysis-deficient subunit (Walker B mutation) activates the ATPase activity of the direct neighbor. One could speculate that this regulatory feature ensures a concerted action of several regulatory functions and oligomerization effects, (ii) observe the influence of arginine mutations in a well defined environment of site-specifically engineered neighboring subunits, and (iii) be independent from overlaying allosteric effects caused by NBD2. In summary, our data indicate that the conserved arginines not only mediate the coupling between nucleotide binding and oligomerization but indeed regulate ATP hydrolysis in a truly allosteric fashion, namely by influencing a catalytic site that they do not directly interact with. Without these arginines, the cooperativity of ATP binding and hydrolysis is completely lost, even if oligomerization is ensured by chemical linkage.

It remains an open question why two such arginines are found in NBD1 of several AAA + proteins, such as ClpB/Hsp104, ClpA, ClpC, and p97/VCP/Cdc48. Wang et al. (19) studied the function of this conserved arginine pair in the N-terminal AAA + domain of p97, which is involved in various cellular processes that are directly or indirectly regulated by the ubiquitin-proteasome system. They also concluded that one arginine is more important for maintaining the hexameric state than the other, but both arginines are essential for intersubunit communication and stimulation of the ATPase activity. Clearly, both arginines are essential and cannot replace each other’s functionality. They may have to work in concert to sense and communicate the nucleotide state and thus facilitate fine tuning of the activity in AAA + protein complexes.

REFERENCES

1. Sanchez, Y., and Lindquist, S. L. (1990) HSP104 required for induced thermotolerance. Science 248, 1112–1115
2. Doyle, S. M., and Wickner, S. (2009) Hsp104 and ClpB: protein disaggregating machines. Trends Biochem. Sci. 34, 40–49
3. Hodson, S., Marshall, J. J., and Burston, S. G. (2012) Mapping the road to recovery: the ClpB/ClpP104 molecular chaperone. J. Struct. Biol. 179, 161–171
4. Zolkiewski, M., Zhang, T., and Nagy, M. (2012) Aggregate reactivation mediated by the Hsp100 chaperones. Arch. Biochem. Biophys. 520, 1–6
5. Tucker, P. A., and Sallai, L. (2007) The AAA+ superfamily: a myriad of motions. Curr. Opin. Struct. Biol. 17, 641–652
6. Weibezahn, J., Tessarz, P., Schleker, 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) Thermotolerance requires refolding of aggregated proteins by substrate translocation through the central pore of ClpB. Cell 119, 653–665
7. Lee, S., Sowa, M. E., Watanabe, Y.-H., Sigler, P. B., Chiu, W., Yoshida, M., and Tsai, F. T. F. (2003) The structure of ClpB: a molecular chaperone that rescues proteins from an aggregated state. Cell 115, 229–240
8. Watanabe, Y. H., Takano, M., and Yoshida, M. (2005) ATP binding to nucleotide binding domain (NBD1) of the ClpB chaperone induces motion of the long coiled-coil, stabilizes the hexamer, and activates NBD2. J. Biol. Chem. 280, 24562–24567
9. Oguchi, Y., Kummer, E., Seyffer, F., Berynskyy, M., Anstett, B., Zahn, R., Wade, R. C., Mogk, A., and Bukau, B. (2012) A tightly regulated molecular toggle controls AAA+ disaggregation. Nat. Struct. Mol. Biol. 19, 1338–1346
10. Sielaff, B., and Tsai, F. T. (2010) The M-domain controls Hsp104 protein remodeling activity in an Hsp70/Hsp90-dependent manner. J. Mol. Biol. 402, 30–37
11. Miot, M., Reidy, M., Doyle, S. M., Hoskins, J. R., Johnston, D. M., Genest, O., Vitye, M. C., Maisson, D. C., and Wickner, S. (2011) Species-specific collaboration of heat shock proteins (Hsp) 70 and 100 in thermotolerance and protein disaggregation. Proc. Natl. Acad. Sci. U.S.A. 108, 6915–6920
12. Rosenzweig, R., Moradi, S., Zarrine-Afsar, A., Glover, J. R., and Kay, L. E. (2013) Unraveling the mechanism of protein disaggregation through a ClpB-Dnak interaction. Science 339, 1080–1083
13. Seyffer, F., Kummer, E., Oguchi, Y., Winkler, J., Kumar, M., Zahn, R., Sourjik, V., Bukau, B., and Mogk, A. (2012) Hsp70 proteins bind Hsp100 regulatory M domains to activate AAA+ disaggregation at aggregate surfaces. Nat. Struct. Mol. Biol. 19, 1347–1355
14. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. I. (1982) Distantly related sequences in the related sequences in the Hsp104 domain of p97, which is involved in various cellular processes that are directly or indirectly regulated by the ubiquitin-proteasome system. They also concluded that one arginine is more important for maintaining the hexameric state than the other, but both arginines are essential for intersubunit communication and stimulation of the ATPase activity. Clearly, both arginines are essential and cannot replace each other’s functionality. They may have to work in concert to sense and communicate the nucleotide state and thus facilitate fine tuning of the activity in AAA + protein complexes.

Acknowledgments—We thank Sabine Zimmermann, Susanne Eisel, and Melanie Müller for excellent technical assistance and acknowledge Susann Mönchgesang for work during a laboratory rotation.

NOVEMBER 21, 2014 • VOLUME 289 • NUMBER 47

JOURNAL OF BIOLOGICAL CHEMISTRY 32975
is not a prerequisite for chaperone activity. J. Biol. Chem. 280, 37965–37973
22. Werbeck, N. D., Kellner, J. N., Barends, T. R. M., and Reinstein, J. (2009) Nucleotide Binding and Allosteric Modulation of the second AAA+ domain of ClpB probed by transient kinetic studies. Biochemistry 48, 7240–7250
23. Werbeck, N. D., Zeymer, C., Kellner, J. N., and Reinstein, J. (2011) Coupling of oligomerization and nucleotide binding in the AAA+ chaperone ClpB. Biochemistry 50, 899–909
24. Zeymer, C., Barends, T. R., Werbeck, N. D., Schlichting, I., and Reinstein, J. (2014) Elements in nucleotide sensing and hydrolysis of the AAA+ H11001 domain of ClpB probed by transient kinetic studies. Acta Crystallogr. D Biol. Crystallogr. 70, 582–595
25. Zeymer, C., Werbeck, N. D., Schlichting, I., and Reinstein, J. (2013) The molecular mechanism of Hsp100 chaperone inhibition by the prion curing agent guanidinium chloride. J. Biol. Chem. 288, 7065–7076
26. Martin, A., Baker, T. A., and Sauer, R. T. (2005) Rebuilt AAA+ motors reveal operating principles for ATP-fuelled machines. Nature 437, 1115–1120
27. Glynn, S. E., Martin, A., Nager, A. R., Baker, T. A., and Sauer, R. T. (2009) Structures of asymmetric ClpX hexamers reveal nucleotide-dependent motions in a AAA+ protein-unfolding machine. Cell 139, 744–756
28. Stinson, B. M., Nager, A. R., Glynn, S. E., Schmitz, K. R., Baker, T. A., and Sauer, R. T. (2013) Nucleotide binding and conformational switching in the hexameric ring of a AAA+ machine. Cell 153, 628–639
29. Schlee, S., Groemping, Y., Herde, P., Seidel, R., and Reinstein, J. (2001) The chaperone function of ClpB from Thermus thermophilus depends on allosteric interactions of its two ATP-binding sites. J. Mol. Biol. 306, 889–899
30. Diemand, A. V., and Lupas, A. N. (2006) Modeling AAA+ ring complexes from monomeric structures. J. Struct. Biol. 156, 230–243
31. Thrall, S. H., Reinstein, J., Wöhrl, B. M., and Goody, R. S. (1996) Evaluation of human immunodeficiency virus type 1 reverse transcriptase primer tRNA binding by fluorescence spectroscopy: specificity and comparison to primer/template binding. Biochemistry 35, 4609–4618
32. Klostermeier, D., Seidel, R., and Reinstein, J. (1999) The functional cycle and regulation of the Thermus thermophilus DnaK chaperone system. J. Mol. Biol. 287, 511–525
33. Hazes, B., and Dijkstra, B. W. (1988) Model building of disulfide bonds in proteins with known three-dimensional structure. Protein Eng. 2, 119–125
34. Werbeck, N. D., Schlee, S., and Reinstein, J. (2008) Coupling and dynamics of subunits in the hexameric AAA+ chaperone ClpB. J. Mol. Biol. 378, 178–190
35. Lee, S., Choi, J.-M., and Tsai, F. T. F. (2007) Visualizing the ATPase cycle in a protein disaggregating machine: structural basis for substrate binding by ClpB. Mol. Cell 25, 261–271
36. Lee, S., Sielaff, B., Lee, J., and Tsai, F. T. (2010) CryoEM structure of Hsp104 and its mechanistic implication for protein disaggregation. Proc. Natl. Acad. Sci. U.S.A. 107, 8135–8140
37. Carroni, M., Kummer, E., Oguchi, Y., Wendler, P., Clare, D. K., Sinning, I., Kopp, J., Mogk, A., Bukau, B., and Saibil, H. R. (2014) Head-to-tail interactions of the coiled-coil domains regulate ClpB activity and cooperation with Hsp70 in protein disaggregation. eLife 3, e02481
38. Wang, F., Mei, Z., Qi, Y., Yan, C., Hu, Q., Wang, J., and Shi, Y. (2011) Structure and mechanism of the hexameric MecA-ClpC molecular machine. Nature 471, 331–335
39. Franzmann, T. M., Czekalla, A., and Walter, S. G. (2011) Regulatory circuits of the AAA+ disaggregate Hsp104. J. Biol. Chem. 286, 17992–18001
40. DeSantis, M. E., Leung, E. H., Sweeny, E. A., Jackrel, M. E., Cushman-Nick, M., Neuhaus-Follini, A., Vashist, S., Sochor, M. A., Knight, M. N., and Shorter, J. (2012) Operational plasticity enables hsp104 to disaggregate diverse amyloid and nonamyloid clients. Cell 151, 778–793
41. Fernández-Higuero, J. A., Acebrón, S. P., Taneva, S. G., Del Castillo, U., Moro, F., and Muga, A. (2011) Allosteric communication between the nucleotide binding domains of caseinolytic peptidase B. J. Biol. Chem. 286, 25547–25555
42. Le, D. T., Eckert, T., and Woehlke, G. (2013) Computer simulation of assembly and co-operativity of hexameric AAA ATPases. PloS One 8, e67815