Allosteric Regulation of Hsp70 Chaperones Involves a Conserved Interdomain Linker*

Received for publication, September 22, 2006 Published, JBC Papers in Press, October 19, 2006, DOI 10.1074/jbc.M609020200

Markus Vogel, Matthias P. Mayer1, and Bernd Bukau2
From the Zentrum für Molekulare Biologie der Universität Heidelberg, 69120 Heidelberg, Germany

The 70-kDa heat shock proteins (Hsp70) are essential members of the cellular chaperone machinery that assists protein-folding processes. To perform their functions Hsp70 chaperones toggle between two nucleotide-controlled conformational states. ATP binding to the ATPase domain triggers the transition to the low affinity state of the substrate-binding domain, while substrate binding to the substrate-binding domain in synergism with the action of a J-domain-containing cochaperone stimulates ATP hydrolysis and thereby transition to the high affinity state. Thus, ATPase and substrate-binding domains mutually affect each other through an allosteric control mechanism, the basis of which is largely unknown. In this study we identified two positively charged, surface-exposed residues in the ATPase domain and a negatively charged residue in the linker connecting both domains that are important for interdomain communication. Furthermore, we demonstrate that the linker alone is sufficient to stimulate the ATPase activity, an ability that is lost upon amino acid replacement. The linker therefore is most likely the lever that is wielded by the substrate-binding domain and the cochaperone onto the ATPase domain to induce a conformation favorable for ATP hydrolysis. Based on our results we propose a mechanism of interdomain communication.

The 70-kDa heat shock protein (Hsp70) chaperones assist de novo folding of newly synthesized polypeptides and disaggregation and refolding of stress-denatured proteins (1, 2). They are involved in the sorting of proteins to different cellular compartments by keeping proteins in a soluble state and assisting translocation across membranes (3–5). Hsp70s also take part in regulation of stability and activity of signal transduction proteins and assembly and disassembly of oligomeric protein structures (6). All of these functions rely on the transient interaction of the C-terminal substrate-binding domain of Hsp70 with short hydrophobic peptide stretches within the substrate polypeptides. This interaction is regulated by the nucleotide status of the ATPase domain such that the affinity of Hsp70 proteins for substrates is low when ATP is bound to their ATPase domain while the affinity is high when no nucleotide or ADP occupies the nucleotide binding pocket. In other words, ATP binding to the ATPase domain controls the conformation of the substrate-binding domain leading to prevalence for the open state of the substrate binding pocket. Vice versa, substrate binding to the substrate-binding domain in synergism with a J-domain protein induces a conformational change in the ATPase domain, thereby stimulating ATP hydrolysis. Substrate-stimulated ATP hydrolysis in return causes a conformational change in the substrate-binding domain, thereby effecting the transition to the high affinity state and the trapping of the substrate.

A number of mutations in both domains of Hsp70 proteins have been isolated that interfere with this interdomain communication mechanism without providing a possible mechanism for the mutual allosteric regulation of the two domains (7–12). The recent structure of a two-domain construct of bovine Hsc70 in the nucleotide-free state shows the contact sites between the ATPase- and substrate-binding domains (13). The interdomain contact is formed by one side of helix A of the substrate-binding domain resting in a groove between subdomains IA and IIA of the ATPase domain (Fig. 1a). How these contacts can subject the ATPase activity to control by substrate binding and how the coupling defects of the previously identified mutations can be rationalized remain unclear. We recently identified in Hsp70 proteins a universally conserved proline in the ATPase domain that acts as molecular switch stabilizing the alternating conformational states in ATPase domain and substrate-binding domain (14). In addition, we identified a likewise universally conserved arginine, Arg151, which is located at the surface of the isolated ATPase domain and is part of a hydrogen bond network connecting this residue via the proline to the catalytic center of the ATPase. Mutational replacement of Arg151 in the Escherichia coli Hsp70 homologue DnaK to alanine caused a complete loss of interdomain communication. These data strongly suggest that Arg151 is part of the mechanics that triggers the conformational changes in the substrate-binding domain upon change of the nucleotide status.

This study was aimed at elucidating the existing gap in our knowledge of the coupling mechanism by identifying residues that continue the signal transduction pathway from the surface of the ATPase domain into the substrate-binding domain, which starts with the aforementioned proline and arginine in the ATPase domain. We found additional surface-exposed residues in the ATPase domain that are not part of the interface
Allosteric Control of Hsp70 Proteins

described in the crystal structure of the two-domain construct of Hsc70 (13) and complementary residues in the conserved linker that have strong effects on interdomain communication when mutationally replaced by other residues. Based on our results we propose a signal transduction mechanism for allosteric regulation in Hsp70 proteins.

EXPERIMENTAL PROCEDURES

Proteins—Site-directed mutagenesis was performed as published (15). Wild-type (DnaKwt) and mutant DnaK were purified according to an established protocol (16) after overproduction in ΔdnaK52 cells (BB1553) (17) to avoid contamination with wild-type protein. Nucleotide bound to DnaK were removed as described earlier (18). The final nucleotide content of the preparations was checked by high pressure liquid chromatography (19) and was generally less than 1%.

Kinetic Measurements—Kinetic measurements were performed in HKM buffer (25 mM HEPES/KOH, pH 7.6, 50 mM KCl, 5 mM MgCl2), at 30 °C. The ATPase activities of DnaKwt and DnaK mutant proteins were determined under single turnover conditions as described (19).

The dissociation rates for peptides were determined as described (20), preincubating 0.5 μM DnaK and 0.5 μM 2-(4’-iodoacetamido)anilino naphthalene-6-sulfonic acid (IAANS)-labeled peptide σ32-Q132-Q144-C-AANS (21) for at least 30 min before mixing with a 50-fold excess of unlabeled σ32-Q132-Q144-C peptide in a PerkinElmer LS55 Luminescence Spectrometer in the absence of ATP and stopped flow instrument (SX.18MV, Applied Photophysics; λex 335 nm, cut-off filter 435 nm) in the presence of ATP.

Fluorescence Spectra—Measurements of the intrinsic tryptophan fluorescence of DnaKwt and DnaK mutant proteins were performed on a PerkinElmer LS-55 Luminescence Spectrometer. Emission spectra of nucleotide-free DnaK (3 μM) in buffer HKM were recorded from 300 to 400 nm at a fixed excitation wavelength of 295 nm at 30 °C in a thermostated cuvette holder. ATP was added to a final concentration of 300 μM. Peptide σ32-Q132-Q144-C was added at 30 μM when indicated.

Circular Dichroism Spectroscopy—DnaKwt and mutant proteins were dialyzed overnight in phosphate buffer (10 mM of the preparations was checked by high pressure liquid chromatography (19). After overproduction with wild-type protein. Nucleotide bound to DnaK were removed as described earlier (18). The final nucleotide content of the preparations was checked by high pressure liquid chromatography (19) and was generally less than 1%.

Circular Dichroism Spectroscopy—DnaKwt and DnaK mutant proteins were determined under single turnover conditions as described (19).

The dissociation rates for peptides were determined as described (20), preincubating 0.5 μM DnaK and 0.5 μM 2-(4’-iodoacetamido)anilino naphthalene-6-sulfonic acid (IAANS)-labeled peptide σ32-Q132-Q144-C-AANS (21) for at least 30 min before mixing with a 50-fold excess of unlabeled σ32-Q132-Q144-C peptide in a PerkinElmer LS55 Luminescence Spectrometer in the absence of ATP and stopped flow instrument (SX.18MV, Applied Photophysics; λex 335 nm, cut-off filter 435 nm) in the presence of ATP.

Fluorescence Spectra—Measurements of the intrinsic tryptophan fluorescence of DnaKwt and DnaK mutant proteins were performed on a PerkinElmer LS-55 Luminescence Spectrometer. Emission spectra of nucleotide-free DnaK (3 μM) in buffer HKM were recorded from 300 to 400 nm at a fixed excitation wavelength of 295 nm at 30 °C in a thermostated cuvette holder. ATP was added to a final concentration of 300 μM. Peptide σ32-Q132-Q144-C was added at 30 μM when indicated.

Circular Dichroism Spectroscopy—DnaKwt and mutant proteins were dialyzed overnight in phosphate buffer (10 mM K2HPO4, pH 7.6) and diluted to a final concentration of 5 μM protein in the same buffer for circular dichroism spectroscopy. The temperature and wavelength scan measurements were performed on a Jasco J750 spectropolarimeter. The proteins were heated up at a rate of 1 degree/min and the circular dichroism measured at 222 nm. At 10, 20, 30, 37, 42, 50, 60, 70, and 85 °C, circular dichroism spectra were recorded from 250 to 190 nm. The melting temperatures Tm for the two temperature transition points were determined by fitting Equation 1

\[
\theta_{222} = \frac{\theta_n + \theta_u \cdot \exp \left[ \frac{\Delta H_u}{RT} \left( 1 - \frac{T}{T_m} \right) + \frac{\Delta C_p}{RT} \cdot \left( T \ln \frac{T}{T_m} + T_m - T \right) \right]}{1 + \exp \left[ \frac{\Delta H_u}{RT} \left( 1 - \frac{T}{T_m} \right) + \frac{\Delta C_p}{RT} \cdot \left( T \ln \frac{T}{T_m} + T_m - T \right) \right]}
\]

(Eq. 1)

(θ222, relative ellipticity at 222 nm; θn and θu, ellipticity of the native and unfolded protein; ΔHu, free enthalpy of unfolding; ΔCp, heat capacity of unfolding; R, universal gas constant) to the unfolding curves between 10 and 60 °C for Tm,1 and between 60 and 85 °C for Tm,2.

RESULTS

Structural Analysis and Mutant Design—To identify residues that are involved in interdomain communication we started with the analysis of the crystal structure of the ATPase domain of bovine Hsc70 centered on the vicinity of residue Arg151,4 which was previously identified to be essential for interdomain communication (14). Because this residue is surface exposed in the ATPase domain, we reasoned that it might directly contact the substrate-binding domain by being part of an interaction surface. We thus inspected the distribution of charges and hydrophobic residues in the neighborhood of Arg151 (Fig. 1b) to characterize the potential interaction surface. The vicinity of this Arg residue has a very particular charge distribution. Three prominent positive charges, Arg, Lys155, and Arg167, in a triangular arrangement are separated by a hydrophobic patch. Although nothing is known so far about Lys155, the residue that corresponds to Arg167 was previously found to be important for the interaction of DnaK with the E. coli J-domain cochaperone, DnaJ. Mutational replacements of this residue in DnaK were able to suppress an aspartic acid to asparagine replacement in the highly conserved and essential His-Pro-Asp (HPD) motif of DnaJ in an allele-specific manner and restore binding of the cochaperone (22). Such an arrangement of positive charges interspaced by hydrophobic residues appears to be complementary to the peptide stretch that connects the ATPase domain of DnaK with the substrate-binding domain, the so-called linker (residues 388–393 in E. coli DnaK), which is highly conserved within the family of Hsp70 proteins. This linker consists of a stretch of four hydrophobic residues (mostly leucine) flanked by two aspartic acid residues (Fig. 1c). Because the hydrophobic residues in the linker were shown to be involved in interdomain communication (12), we hypothesized that the negatively charged residues of the linker and the positive charges of the ATPase domain may interact and be involved in the transmission of the signals between the two domains. We therefore replaced in DnaK Lys155 by alanine (K155A) and aspartic acid (K155D), Arg167 by alanine (R167A) and aspartic acid (R167D), Asp388 by arginine (D388R), and Asp393 by arginine (D393R) and alanine (D393A) and measured the properties of the resulting mutant proteins. In addition, we compared two ATPase domain fragments, one ending before the linker (DnaK-(2–385)) and a second including the linker (DnaK-(2–393)), such as to analyze effects of the linker on the isolated ATPase domain.

Except for the dnaK-K155A and the dnaK-D388R alleles, none of the mutant alleles was able to complement the temperature-sensitive phenotype of a ΔdnaK strain in vivo (Table 1). The structural integrity of the mutant proteins was verified by circular dichroism spectroscopy at temperatures between 10 and 85 °C. The thermal unfolding transitions of all mutant pro-

4 Numbering throughout this report is according to E. coli DnaK; Arg151 in DnaK corresponds to Arg155 in bovine Hsc70; Lys155 to Lys159, Arg167 to Arg171, Pro143 to Pro147, Asp388 to Asp390, Asp393 to Asp395, and Arg171 to Arg175.
sequences relative to Hsp70 sequences. The presentation of the electrostatic surface potential of the ATPase domain of bovine Hsc70 (Protein Data Bank entry code 1DKZ (30), residues 513–603). The ATPase domain is in blue, and the dark blue line indicates the position of the linker in the alternative conformation (30). The residues important for this study are shown in stick representation. The lower panel shows the same structure as the upper panel rotated by ~90° as indicated. The image was created in PyMOL (www.pymol.org).

TABLE 1

| DNA sequence analysis of representative, evolutionary distant Hsp70 proteins based on the crystal structure of the two-domain construct of bovine Hsc70 (Protein Data Bank entry code 1YUW (13), residues 1–518) and the crystal structure of the substrate-binding domain of DnaK (Protein Data Bank entry code 1DKZ (30), residues 513–603). The ATPase domain is in orange, the substrate-binding domain in blue, and the dark blue line indicates the position of the linker in the alternative conformation (30). The residues important for this study are shown in stick representation. The lower panel shows the same structure as the upper panel rotated by ~90° as indicated. The image was created in PyMOL (www.pymol.org)., b, represen-
| Complication of the temperature sensitivity of a Δ dnaK E. coli strain (BB1553 (17) with plasmid-encoded IPTG-inducible dnaK alleles. 10-fold dilutions of an overnight culture were spotted onto Luria Bertani plates containing the indicated IPTG concentrations. The plates were subsequently incubated overnight at 30 and 40 °C. The plating efficiencies are calculated from the colonies at 40 °C at the given IPTG concentration divided by the colonies at 30 °C at 0 μM IPTG. —, plating efficiency smaller than 10−4.
| | IPTG (μM) | 0 | 50 | 100 | 150 | 250 |
| dnaKwt | | 0.03 | 1 | 1 | 0.05 |
| Vector | | — | — | — | — | — |
| dnaK-K155A | | 0.0006 | — | 0.006 | 0.2 | — |
| dnaK-K155D | | — | 0.01 | — | — | — |
| dnaK-R167A | | — | — | — | — | — |
| dnaK-R167D | | — | — | — | — | — |
| dnaK-R388R | | — | — | — | — | — |
| dnaK-D393A | | — | — | — | — | — |
| dnaK-D393R | | — | — | — | — | — |
| dnaK-D393K | | — | — | — | — | — |
| dnaK-K155D,D393R | | — | — | — | — | — |
| dnaK-R167D,D388R | | — | — | — | — | — |
| dnaK-D393A,D393K | | — | — | — | — | — |

Effect of the Amino Acid Replacements on ATP-stimulated Substrate Dissociation—Consistent with earlier publications, a fluorescent-la
eled peptide dissociates from wild-
type DnaK (DnaKwt) with a rate of ~10−13 s−1 in the absence of nucleotides (20). The presence of ATP leads to a 1000-fold increase in the dissociation rate to ~0.9 s−1. As shown in Fig. 2, all amino acid replacements except in position 388 lead to a significant reduction of ATP-stimulated substrate release, indicating that positions 155, 167, and 393 are instrumental for interdomain communication. In each case, charge reversal had a more dramatic effect than charge neutralization by replacement with alanine, suggesting that electrostatic forces are important for the coupling status (Fig. 2, lower panel). Combination of charge reversal in position 155 or 167 with charge reversal in position 393 (DnaK-K155D,D393R and DnaK-R167D,D393R) did not restore the loss of ATP-stimulated substrate release (data not shown).

Interdomain Communication Assessed by Tryptophane Fluorescence—To further analyze the effects of the amino acid replacements, we determined the coupling status of the ATPase- and substrate-binding domain by measuring the fluorescence emission maximum of the single tryptophane residue positioned in the ATPase domain of DnaK. Upon addition of ATP, tryptophane fluorescence in DnaKwt displays a blueshift of its emission maximum by 3 to 4 nm. This effect is dependent on the presence of the substrate-binding domain and on intact allosteric coupling of the domains (24, 25). The fluorescence emission maximum of all mutant proteins corresponded to that of DnaKwt in the nucleotide-free state. Addition of ATP induced a wild-type-like blueshift in DnaK-D388R and a slightly reduced blueshift in DnaK-K155A and DnaK-R167A, while DnaK-K155D, DnaK-R167D, and the variants with replacements in position 393 exhibited a blueshift below 1 nm or no blueshift at all (Fig. 3, black bars). For DnaK-R167D, DnaK-D393A, and DnaK-D393R this result is consistent with the substrate dissociation kinetics. In these variants substrate release was stimulated by ATP only very slightly, indicating an almost complete loss of interdomain communication. The tryptophane fluorescence data for DnaK-D388R are also consistent with the substrate dissociation kinetics because in this mutant protein substrate release was stimulated by ATP as in DnaKwt, demonstrating a coupled status for this protein. In contrast, the tryptophane fluorescence data for DnaK-K155A proteins were found to be similar to that of the wild-type protein (Table 2).

We analyzed interdomain communication by three assays: 1) stimulation of substrate release by ATP (23); 2) intrinsic fluores-
cence of the single tryptophane located in the ATPase domain, which was shown to be responsive to conformational changes in

interdomain communication.

FIGURE 1. Structural basis for the mutant design. a, secondary structure representation of Hsp70 proteins based on the crystal structure of the two-domain construct of bovine Hsc70 (Protein Data Bank entry code 1YUW (13), residues 1–518) and the crystal structure of the substrate-binding domain of DnaK (Protein Data Bank entry code 1DKZ (30), residues 513–603). The ATPase domain is in orange, the substrate-binding domain in blue, and the dark blue line indicates the position of the linker in the alternative conformation (30). The residues important for this study are shown in stick representation. The lower panel shows the same structure as the upper panel rotated by ~90° as indicated. The image was created in PyMOL (www.pymol.org).
We observed earlier that a bound peptide substrate can influence tryptophane fluorescence profoundly via an effect on the coupling status (14). Binding of a substrate peptide decreases the blueshift of wild-type DnaK by ~1 nm in contrast to a variant protein with defects in interdomain communication that shows an increase in blueshift upon addition of a peptide (27). We therefore measured the blueshift of tryptophane fluorescence for the DnaK variants in the presence of a substrate peptide that did not contain any tryptophane residues. In the presence of the substrate peptide the blueshift of tryptophane fluorescence was completely abolished in DnaK-K155A, DnaK-K155D, and DnaK-R167A variant proteins, indicating a severe impairment of interdomain communication (Fig. 3, gray bars). Taken together, tryptophane fluorescence confirmed the importance of residues Lys155, Arg167, and Asp393 for the coupling of ATPase- and substrate-bind domains.

**Effect of the Amino Acid Replacements on ATPase Activity**—To analyze the effects of the amino acid replacements on the substrate- and DnaJ-mediated control of the ATPase activity of DnaK, the ATP hydrolysis rates of wild-type and mutant DnaK proteins were measured under single turnover conditions in the absence and presence of DnaJ and the E. coli heat shock transcription factor σ32 as native protein substrate of DnaK. For comparison the data for the DnaK-R151A mutant are also shown (taken from Ref. 27). As published earlier, DnaKwt hydrolyzes ATP with a rate of $6 \times 10^{-4} \text{s}^{-1}$ (28). Separate addition of σ32 (1 μM) and of DnaJ at low, physiological concentrations (50 nM) accelerates ATP hydrolysis by a factor of 2.0 and 2.7, respectively. Simultaneous addition of DnaJ and σ32 synergistically stimulates the ATPase rate of DnaK by 50-fold (12).

Except for the Asp388 to Arg replacement, all amino acid replacements caused an increase in basal ATP hydrolysis activity of DnaK by 3-fold (R167A) to 12-fold (D393A) (Fig. 4, a and c). The amino acid replacements had opposing effects on the DnaJ-mediated stimulation of DnaK ATPase activity. The stimulation of the ATPase activity by 50 nM DnaJ was very low (1.3- to 1.5-fold over the individual basal rates) when residues in position 167 and 393 were replaced. In contrast, the stimulation factor was increased 2- and 3-fold over the stimulation factor for DnaKwt in the DnaK-K155A and D388R mutant proteins (Fig. 4b). The ATPase stimulation by the protein substrate σ32 was approximately normal for DnaK-R167A, DnaK-K155A, DnaK-K155D, and DnaK-D388R but completely lost for DnaK-R167D, DnaK-D393R, and DnaK-D393A. The absolute value of the synergistically stimulated ATPase activity of DnaK-D388R by the simultaneous presence of DnaJ and σ32 (Fig. 4a) as well as the stimulation factor over the individual basal rate (Fig. 4b) were reduced by a factor of 2 as compared with wild-type DnaK. In contrast, DnaK-K155A and DnaK-K155D exhibited only a slight reduction in the absolute value of the synergistically stimulated ATPase rate but a greatly reduced value for the stimulation factor over the basal rate. The synergistic stimulation of the ATPase activity was greatly reduced for DnaK-R167A and completely absent for DnaK-R167D and the variants with replacements in position 393 (Fig. 4). These data suggest that Lys155 and Arg167 in the ATPase domain and Asp393 in the linker are involved in

| TABLE 2 Stability of the mutant proteins is not severely compromised by the mutational alterations |
|---------------------------------|----------|----------|
|                               | $T_{m,1}$ | $T_{m,2}$ |
| DnaKwt                         | 41.6 ± 0.2 | 75.5 ± 0.3 |
| DnaK-K155A                     | 43.5 ± 0.2 | 75.6 ± 0.2 |
| DnaK-K155D                     | 47.4 ± 0.1 | 74.4 ± 0.2 |
| DnaK-R167D                     | 45.9 ± 0.2 | 75.7 ± 0.5 |
| DnaK-R167A                     | 44.7 ± 0.2 | 75.7 ± 0.5 |
| DnaK-D388R                     | 43.8 ± 0.2 | 75.2 ± 0.4 |
| DnaK-D393R                     | 45.8 ± 0.3 | 75.7 ± 0.5 |
| DnaK-D393A                     | 42.2 ± 0.2 | 75.3 ± 0.4 |
| DnaK-K155D,D393R               | 47.6 ± 0.1 | 74.5 ± 0.2 |
| DnaK-R167D,D388R               | 46.7 ± 0.1 | 74.8 ± 0.2 |
| DnaK-R167D,D393R               | 46.3 ± 0.2 | 74.5 ± 0.3 |

and DnaK-R167A seem to contradict the substrate dissociation results. Whereas tryptophane fluorescence blueshift was only reduced slightly, ATP-stimulated substrate release was severely impaired in these mutant proteins (compare Figs. 2 and 3).
interaction with DnaJ and interdomain communication, whereby charge reversal in position 167 again had a stronger effect than neutralization.

Effect of the Linker on the ATPase Activity—The comparison of the ATPase activities of the ATPase domain fragment ending just before the linker (DnaK-(2–385)) with an ATPase domain construct that included the linker (DnaK-(2–393)) yielded a surprising result. Whereas DnaK-(2–385) had a basal ATPase activity that was slightly lower than the basal ATPase activity of wild-type full-length DnaK, DnaK-(2–393) had a basal ATPase activity that was 41-fold higher and therefore almost as high as the stimulated ATPase activity of full-length DnaKwt in the simultaneous presence of DnaJ and σ^32 (Fig. 4c). The ATPase activities of both ATPase domain constructs were not influenced by DnaJ (not shown). This increase of the ATPase activity by the linker was lost if the highly conserved hydrophobic residues of the linker were replaced by alanines (DnaK-(2–393)A4, DnaK-(2–393)V389A, L390A, L391A, L392A) and reduced to the basal rate of the full-length protein with the similar replacement when the last residue Asp^393 was replaced by Ala or Arg (Fig. 4c, DnaK-(2–393)D393A, DnaK-(2–393)D393R). These data clearly demonstrate that the linker by itself can stimulate the ATPase activity of the ATPase domain of DnaK and that mutational replacements that in the full-length protein lead to a loss of substrate and DnaJ-mediated stimulation of the ATPase activity also obliterate the linker effect.

To determine whether the linker acts through the positively charged residues Arg^151, Lys^155, or Arg^167 as originally hypothesized, ATPase domain constructs including the linker with replacements of these residues were analyzed (Fig. 4c). Surprisingly, all three constructs tested, DnaK-(2–393)R151A, DnaK-(2–393)K155D, and DnaK-(2–393)R167D exhibited a further increased ATPase rate close to the limits measurable by hand pipetting and 2- to 3-fold over the rate measured for DnaKwt synergistically stimulated by 1 μM σ^32 and 50 nM DnaJ. These data suggest that the linker is responsible for triggering ATP hydrolysis while the positively charged residues in the ATPase domain negatively modulate the ATPase activity.

DISCUSSION

In this study we demonstrated that two positively charged residues at the surface of subdomain IA in the ATPase domain and a negatively charged residue in the linker are instrumental for mutual allosteric regulation of the ATPase- and substrate-binding domains. Furthermore, our data show that interaction of the linker itself with the ATPase domain triggers ATP hydrolysis and is therefore responsible for transmitting the signal of the bound substrate to the ATPase domain. Based on these results we propose that the linker is a central part of the mechanism that couples ATP binding and hydrolysis to conformational changes in the substrate-binding domain.

The involvement of linker residues 389–393 in interdomain communication is now clearly established. We show in this study unambiguously that the aspartic acid residue in position 393 is essential for interdomain communication. First, the replacement of this residue by alanine or arginine abolishes the ATP stimulation of the substrate dissociation rate. A model peptide substrate still binds to DnaK-D393R and DnaK-D393A and is released in the absence of nucleotide with a rate similar to DnaKwt. The addition of ATP has within the error range of the experiment no effect on substrate dissociation from DnaK-
Allosteric Control of Hsp70 Proteins

D393R and very little effect on substrate dissociation from DnaK-D393A (7-fold stimulation as compared with a 1000-fold stimulation of DnaKwt). Second, the fluorescence emission maximum of the single tryptophane in the ATPase domain is not influenced by ATP binding. Third, no synergistic stimulation of the single turnover ATPase rate by DnaJ and the protein substrate $\sigma^{32}$ is observable. Asp$^{393}$ is highly conserved within the Hsp70 proteins, indicating evolutionary pressure against replacement. Even glutamates are not found in this position within the Hsp70 proteins (Fig. 1). It is also conserved in the Hsp70-related Hsp110 and Hsp170 proteins but in some members of these families is replaced by glutamate. Interestingly, Asp$^{393}$ is not found in Hsp70-related proteins without a classical substrate-binding domain like Saccharomyces cerevisiae Ssz and Homo sapiens STCH.

The properties of Asp$^{393}$ are shared by the adjacent, similarly conserved hydrophobic residues in positions 389–392, which were shown earlier to be essential for interdomain communication (12). Mutant proteins with alanine replacements of all four hydrophobic residues or aspartic acid replacements of two of the four residues (DnaK-L390D, L391D) lack all of the three above-mentioned signs of interdomain communication. The contribution of the hydrophobicity of the linker residues was contested by a more recent publication (29) reporting that the substitution of two of the four hydrophobic residues for glycine and serine (DnaK-L391G, L392S) did not obliterate interdomain communication completely. This suggests that not all four hydrophobic residues are essential for domain-domain coupling and that the results of the DnaK-L390D, L391D variant is more likely due to the surplus of two negative charges than to the loss of hydrophobicity within the linker.

Another conserved residue of the linker, the negatively charged residue Asp$^{388}$, does not play such an important role in interdomain communication because its replacement by the positively charged arginine had only minor effects on the synergistic stimulation of the ATPase activity by DnaJ and $\sigma^{32}$ and no effects on ATP-stimulated substrate release and tryptophane fluorescence. These results are consistent with earlier observations (29). The comparison of the results with DnaK-D388R and DnaK-D393R demonstrates that charge reversal in this region of the protein is not sufficient for interruption of interdomain communication but that position 393 is very specific in this respect.

In the crystal structure of the substrate-binding domain of DnaK the linker was found in two different positions, in one position extended and freely exposed to the solvent and in another folded back toward the base of the $\alpha$-helix A and bound to a hydrophobic groove that is formed between the two $\beta$-sheets (30). These two different conformations of the linker do not seem to be crystallization artifacts but in fact part of the nucleotide-induced conformational changes. In a recent amide hydrogen exchange study we provide evidence that the amide protons of the linker are completely exposed in the nucleotide-free state and become inaccessible to solvents when ATP is bound to the ATPase domain (31). Interestingly, substrate binding to DnaK-ATP reverts this ATP-induced protection of the linker. The point in the crystal structure of the substrate-binding domain where the linker bends into the two alternative conformations is exactly at Asp$^{393}$ (30). If these two alternative conformations are linked to interdomain communication it is likely that interactions with Asp$^{393}$ would be crucial for the conformational transitions and an amino acid replacement at this position would be detrimental to interdomain communication. Such a mechanism would explain the dramatic effect of the D393A replacement. In addition, in the bound position two of the leucine side chains, Leu$^{390}$ and Leu$^{391}$, are buried in a hydrophobic groove within the substrate-binding domain, while the other two hydrophobic residues, Val$^{389}$ and Leu$^{391}$, are not resolved in the crystal structure beyond the C$\beta$, indicating solvent exposure and flexibility. We originally assumed that these two hydrophobic residues would also interact with the hydrophobic patch in the ATPase domain near Arg$^{151}$ and that the negatively charged Asp$^{388}$ and Asp$^{393}$ would interact with two of the three surrounding positively charged residues, Arg$^{151}$, Lys$^{155}$, and Arg$^{167}$. Although all three positively charged residues are important for interdomain communication as shown in this work and in an earlier publication (14), the recent crystal structure of a two-domain construct of bovine Hsc70 makes such an interaction very unlikely because the respective residues are too far apart from each other in this structure (13). However, the ATPase domain including the linker exhibits a fully stimulated ATPase activity, whereas the ATPase domain without the linker has only the very low ATPase activity of the unstimulated full-length protein. This fact provides solid evidence that the linker also interacts with the ATPase domain and somehow alters its conformation, bringing the catalytic residues into a more ideal position for ATP hydrolysis. The hydrophobic residues of the linker (Val$^{389}$ to Leu$^{392}$) and Asp$^{393}$ seem to be important for this interaction because a replacement of these residues in the ATPase domain-linker construct reduces the stimulatory effect of the linker to the basal rate of a full-length protein with the same mutations. How the linker stimulates the ATPase activity is not clear. It does not seem to interact with positively charged residues investigated in this study as originally hypothesized, because replacement of Arg$^{151}$ by alanine or Lys$^{155}$ and Arg$^{167}$ by aspartates does not diminish but in contrast enhances the linker effect on the ATPase activity.

The question remains how Lys$^{155}$ and Arg$^{167}$ contribute to interdomain communication. Both residues are solvent exposed in all crystal structures available, including the new structure of the two-domain construct of Hsc70, without any functional groups nearer than 6 Å to their side chains. Nevertheless, replacement of either residue by alanine reduces the ATP-stimulated substrate dissociation rate to 1/30th of the rate of DnaKwt, obliterates the ATP-induced blueshift in the presence of a peptide substrate, and reduces the DnaJ- and $\sigma^{32}$-stimulated ATPase rate by 85 to 90%, respectively. The reduction of the DnaJ- and $\sigma^{32}$-stimulated ATPase rate could be due to a failure of DnaK-R167A to interact with DnaJ as reported earlier (22). However, the effect on ATP-stimulated substrate release and ATP-induced blueshift of tryptophane fluorescence cannot be explained in this way, because DnaJ is not involved in these in vitro reactions. It is particularly interesting that an ATP-induced blueshift was observed in DnaK-K155A and DnaK-R167A in the absence of a substrate peptide but not in its pres-
ence. Such an observation was not made with any other mutant protein investigated in our laboratory so far and, in fact, contrasts observations with replacement variants of Pro\textsuperscript{143} (14). DnaK-P143A and DnaK-P143G showed only a small blueshift in the absence of a peptide substrate but an almost wild-type-like blueshift in the presence of a substrate peptide. These results argue for a direct link between the substrate binding pocket and Lys\textsuperscript{155} and Arg\textsuperscript{167}, which is disrupted by the alanine replacements. In other words, in the absence of a substrate peptide the two domains of the replacement variants still communicate with each other and ATP binding can induce conformational changes in the substrate-binding domain leading to an opening of the lid over the substrate binding pocket, which affects the environment of Trp\textsuperscript{102} in the ATPase domain. Peptide binding, however, induces a conformational change in the substrate-binding domain that may weaken some links between the domains and thereby make interdomain communication much more dependent on interactions with Lys\textsuperscript{155} and Arg\textsuperscript{167} in the ATPase domain. Taken together, these data suggest multiple interaction points between the two domains that are important for domain-domain coupling. At least Lys\textsuperscript{155} would not have been suspected to contribute to interdomain communication based on the recent crystal structure of the two-domain construct of bovine Hsc70 (13), indicating that additional structural information is needed to fully understand interdomain communication.

How are these residues linked to the ATP-binding site? Lys\textsuperscript{155} is situated in the same α-helix as Arg\textsuperscript{151}, which was earlier shown to be essential for interdomain communication and suggested to act as a relay that transmits the ATP binding signal to the substrate-binding domain (14). A force acting on Lys\textsuperscript{155} could induce a slight rotation of the helix and thereby affect the position of Arg\textsuperscript{151}. Arg\textsuperscript{167} is also connected to the structure that is proposed to transmit the ATP binding signal to the substrate-binding domain. In the crystal structure of bovine Hsc70 ATPase domain (Protein Data Bank entry 1HPM) the carbonyl oxygen of Arg\textsuperscript{167} (Arg\textsuperscript{171} in Hsc70) is hydrogen bonded with the amide of Val\textsuperscript{141} (Val\textsuperscript{144} in Hsc70). The β-strand (residues 164–169) on which Arg\textsuperscript{167} is positioned forms a sheet with the β-strand (residues 138–141) that immediately precedes Pro\textsuperscript{143}. This proline constitutes the molecular switch that stabilizes two alternating conformations of Hsp70 proteins corresponding to the ATP-bound low affinity state and the ADP-bound high affinity state (14). In response to ATP binding this residue was proposed to undergo conformational changes, thereby shifting the position of the highly conserved loop Pro\textsuperscript{143}-Phe\textsuperscript{146} and Arg\textsuperscript{151} consistent with changes in chemical shifts that were observed earlier by NMR in these residues (32). Such conformational changes may be affected by the preceding β-sheet and Arg\textsuperscript{167} may act through this β-sheet.

Based on our data, the recent amide hydrogen exchange study (31), and earlier structural and biochemical studies, we propose that ATP binding to the ATPase domain causes a bending of the linker at position 393 with the consequence of inserting it between the ATPase domain and substrate-binding domain. This movement triggers the low affinity state of the substrate-binding domain. The linker, however, does not contact the ATPase domain in the optimal position for induction of ATP hydrolysis. Substrate and DnaJ binding lead to a repositioning of the linker, which in turn causes a conformational change in the ATPase domain, most likely involving Lys\textsuperscript{155}, Arg\textsuperscript{167}, and Arg\textsuperscript{151}, which finally triggers the proline switch to transit into the conformation optimal for ATP hydrolysis.

REFERENCES

1. Bukau, B., Deuerling, E., Pfund, C., and Craig, E. A. (2000) Cell 101, 119–122
2. Hartl, F. U., and Hayer-Hartl, M. (2002) Science 295, 1852–1858
3. Young, J. C., Agashe, V. R., Siegers, K., and Hartl, F. U. (2004) Nat. Rev. Mol. Cell. Biol. 5, 781–791
4. Neupert, W., and Brunner, M. (2002) Nat. Rev. Mol. Cell. Biol. 3, 555–565
5. Ryan, M. T., and Pflanzer, N. (2002) Adv. Protein Chem. 59, 223–242
6. Wegele, H., Muller, L., and Buchner, J. (2004) Rev. Physiol. Biochem. Pharmacol. 151, 1–44
7. O’Brien, M. C., and McKay, D. B. (1993) J. Biol. Chem. 268, 24323–24329
8. Montgomery, D. L., Morimoto, R. I., and Gierasch, L. M. (1999) J. Biol. Chem. 274, 915–932
9. Wei, J., and Hendershot, L. M. (1995) J. Biol. Chem. 270, 26670–26676
10. Johnson, E. R., and McKay, D. B. (1999) Biochemistry 38, 10823–10830
11. Sousa, M. C., and McKay, D. B. (1998) Biochemistry 37, 15392–15399
12. Laufen, T., Mayer, M. P., Beisel, C., Klostermeier, D., Reinstein, J., and Bukau, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5452–5457
13. Jiang, J., Prasad, K., Lafer, E. M., and Sousa, R. (2005) Mol. Cell 20, 513–524
14. Vogel, M., Bukau, B., and Mayer, M. P. (2006) Mol. Cell 21, 359–367
15. Kunkel, T. A., Bebenek, K., and McClary, J. (1991) Methods Enzymol. 204, 125–139
16. Buchberger, A., Schröder, H., Büttrn, M., Valencia, A., and Bukau, B. (1994) Nat. Struct. Biol. 1, 95–101
17. Bukau, B., and Walker, G. (1990) EMBO J. 9, 4027–4036
18. Theyssen, H., Schuster, H.-P., Bukau, B., and Reinstein, J. (1996) J. Mol. Biol. 263, 657–670
19. Mayer, M. P., Laufen, T., Paal, K., McCarty, I. S., and Bukau, B. (1999) J. Mol. Biol. 289, 1131–1144
20. Mayer, M. P., Schröder, H., Rüdiger, S., Paal, K., Laufen, T., and Bukau, B. (2000) Nature Struct. Biol. 7, 586–593
21. McCarty, J. S., Rüdiger, S., Schönfeld, H.-J., Schneider-Mergener, J., Nakahigashi, K., Yura, T., and Bukau, B. (1996) J. Mol. Biol. 256, 829–837
22. Sult, W.-C., Burkholder, W. F., Lu, C. Z., Zhao, X., Gottesman, M. E., and Gross, C. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15223–15228
23. Schmidt, D., Baici, A., Gehring, H., and Christen, P. (1994) Science 263, 971–973
24. Buchberger, A., Theyssen, H., Schröder, H., McCarty, I. S., Virgallita, G., Milkreit, P., Reinstein, J., and Bukau, B. (1995) J. Biol. Chem. 270, 16903–16910
25. Moro, F., Fernandez, V., and Muga, A. (2003) FEBS Lett. 533, 119–123
26. Karzai, A. W., and McMacken, R. (1996) J. Biol. Chem. 271, 11236–11246
27. Vogel, M., Bukau, B., and Mayer, M. P. (2006) Mol. Cell 21, 359–367
28. McCarty, J. S., Buchberger, A., Reinstein, J., and Bukau, B. (1995) J. Mol. Biol. 249, 126–137
29. Han, W., and Christen, P. (2001) FEBS Lett. 497, 55–58
30. Zhu, X., Zhao, X., Burkholder, W. F., Gragerov, A., Ogata, C. M., Gottesman, M., and Hendrickson, W. A. (1996) Science 272, 1606–1614
31. Rist, W., Graf, C., Bukau, B., and Mayer, M. P. (2006) J. Biol. Chem. 281, 16493–16501
32. Revington, M., Holder, T. M., and Zuiderweg, E. R. (2004) J. Biol. Chem. 279, 33958–33967

5 M. Vogel, B. Bukau, and M. P. Mayer, unpublished data.