Amide Hydrogen Exchange Reveals Conformational Changes in Hsp70 Chaperones Important for Allosteric Regulation*

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Hsp70 chaperones assist protein folding processes by a nucleotide-driven cycle of substrate binding and release. Although structural information is available for the isolated nucleotide-binding (NBD) and substrate-binding domains (SBD) in the high affinity conformation, the low affinity conformations and the conformational changes associated with mutual allosteric regulation remained largely enigmatic. By using amide hydrogen exchange in combination with mass spectrometry, we analyzed the Escherichia coli Hsp70 homologue DnaK as full-length protein and its individual domains in the nucleotide-free and ATP-bound conformation. We found a surprising degree of flexibility in both domains. The comparison of the full-length protein with the isolated domains demonstrates a mutual stabilization of both domains. This protection from solvent was most pronounced and in addition was nucleotide-dependent in the lower β-sheet of the SBD and the loop that connects the last β-strand with helix αA. Interestingly, the linker region, which connects NBD and SBD and which is close to the protected loop in the SBD, is solvent-exposed in the absence of nucleotide and completely protected from hydrogen exchange in the presence of ATP. Peptide binding to DnaK-ATP reverts the ATP-induced conformational changes in the linker and selected parts of the NBD. Our data outline a pathway for allosteric interdomain control and suggest an important role of the linker and the base of helix αA.

Hsp70 chaperones assist a large variety of cellular protein folding processes ranging from folding of newly synthesized and stress-denatured polypeptides to the control of activity and stability of many native regulatory proteins (1, 2). All of these functions are performed by Hsp70 chaperones through nucleotide-regulated transient interactions with their substrates. Hsp70 proteins consist of an N-terminal nucleotide-binding domain (NBD)5 with an actin-like fold connected via a short, highly conserved linker to a C-terminal, structurally unique substrate-binding domain (SBD). The SBD is further subdivided in a α-helix/β-sheet subdomain containing the substrate-binding pocket and a helical subdomain considered to function as a lid over the substrate-binding pocket (Fig. 1c) (3, 4). The substrate peptide, cocristallized in the SBD, was bound by hydrogen bonds to the peptide backbone and by hydrophobic interactions mainly between a single leucine of the substrate and the substrate-binding pocket. The crystal structure of the SBD in the high affinity conformation made clear that conformational changes are necessary to allow binding and release of substrates in this state. However, which parts of the SBD move to allow substrate binding remained elusive.

ATP binding at the bottom of a deep cleft in the NBD induces conformational changes in the SBD leading to the transition from the high affinity to the low affinity state. Conversely, substrate binding to the SBD in synergism with a cochaperone of the J-domain protein family triggers ATP hydrolysis in the NBD. How the mutual allosteric regulation is achieved and what the conformational changes are with which this regulation is associated remained largely enigmatic because of the lack of suitable structural information. The crystal structure of the NBD of wild-type and mutant bovine Hsc70 and human Hsp70 proteins in complex with different nucleotides did not reveal major nucleotide-dependent conformational changes (5–8). NMR investigations on the NBDs of bovine Hsc70 and Thermus thermophilus DnaK suggested some flexibility and a shearing movement of the subdomains of the NBD and show nucleotide-dependent chemical shift perturbations (9, 10). How these perturbations relate to conformational changes and allosteric regulation, however, remained largely unclear. More recently, the crystal structure of a two-domain construct of bovine Hsc70 in the nucleotide-free state containing the NBD, the β-sheet subdomain, and part of the helical subdomain was published showing the contact sites between NBD and SBD (11). However, which conformational changes are linked to the ATPase cycle of Hsp70 proteins is still enigmatic.

To analyze the conformational dynamics in Hsp70 proteins that allow substrate binding in the high affinity state and the nucleotide-dependent conformational change, we used native state amide hydrogen exchange (HX) technology in combination with mass spectrometry (MS), and we mapped the solvent accessibility of the backbone amides in Escherichia coli DnaK in dependence of bound nucleotide.

EXPERIMENTAL PROCEDURES

Proteins—Wild-type DnaK, DnaK-T199A, DnaK-R151A, and DnaK-2–385 were purified and made nucleotide-free as described (12, 13). The SBD of DnaK was purified as N-terminally histidine-tagged protein on Ni2+-nitrilotriacetic acid fast flow Sepharose and subsequent ion exchange chromatography.

Mass Spectrometry and Data Processing—Electrospray ionization-mass spectra were acquired on a quadrupole time of flight instrument (QSTAR pulsar, Applied Biosystems). Protein mass spectra were deconvoluted using the BioAnalyst software (Applied Biosystems). For full-length proteins, spectra were externally calibrated using apomyoglobin. Peptides of DnaK were identified either by their MS/MS spectra or by their exact masses. The deuterium content of the peptides was calculated by using the average mass difference between the isotopic env-
lapses of deuterated and undeuterated peptides. The average masses were determined by the MagTran software (14).

**HX Experiments**—Nucleotide-free DnaK proteins were preincubated with an excess of ATP for 1 min or a peptide substrate (1/H9268 32-Met195–Asn207 (15)) for 1 h at 30 °C.

HX experiments were performed similar to those described earlier (16). Amide hydrogen exchange was initiated by a 20-fold dilution of 200 pmol of DnaK into D2O containing 25 mM HEPES, pH 7.6, 50 mM KCl, and 5 mM MgCl2 at 30 °C. After various time points (10 s-90 min), exchange reaction was quenched by decreasing the temperature to 0 °C and the pH with quench buffer (400 mM KH2PO4/H3PO4, pH 2.2). Quenched samples were directly injected into an HPLC setup and analyzed on an electrospray ionization-quadrupole time of flight-mass spectrometer (QSTAR Pulsar, Applied Biosystems) as described (17).

For full-length protein analysis samples were trapped on a reversed-phase column (1/H11003 20 mm; POROS 10 R 1 media, PerSeptive Biosystems), desalted for 1 min with buffer A (0.05% trifluoroacetic acid in H2O) at 400 l/min for 1, and eluted with 80% buffer B (90% acetonitrile, 0.05% trifluoroacetic acid) at 20 l/min for analysis. All HPLC components that come into contact with the samples are immersed in an ice-bath to minimize back exchange.

For the analysis of peptide fragments samples were mixed 1:1 with quench buffer additionally containing 2M guanidinium hydrochloride. The HPLC setup contained in these cases a combination of two columns (2 × 20 mm) packed with pepsin and Protease XIII (Aspergillus saitoi) immobilized on POROS-20AL beads (PerSeptive Biosystems). The resulting peptides were trapped on a reversed-phase column (POROS 50 R 2, PerSeptive Biosystems) and eluted from the trap column over a 0.75-mm inner diameter × 6-cm analytical reversed-phase column packed with Zorbax 300SB-C8 (3.5-μm particles) with a 10-min gradient of 15–55% buffer B into the electrospray source. The whole setup was immersed in an ice-bath to minimize back-exchange. A fully deuterated DnaK sample was analyzed under the same conditions to correct for back-exchange.

**Calculation of the Global Protection Factor**—Experimental data were fit by nonlinear least squares to a sum of three exponential terms resulting in three exchange rates with the amplitudes corresponding to the number of exchanging amide hydrogens. The average chemical exchange rate was determined by simulating deuteron incorporation for different time intervals and back-exchange during the desalting step using the exchange rates for pD 7.6 and pH 2.4 calculated with the HXPep program (courtesy Z. Zhang) and fitting a single exponential equation to the values. Protection factors were calculated according to the equation for the EX2 exchange regime, 

\[
G = \frac{1}{K_{unf}} \frac{k_{ch}}{k_{obs}} \cdot \ln \frac{k_{obs}}{k_{ch}}
\]

where \(K_{unf}\) indicates the unfolding equilibrium constant; \(k_{ch}\) indicates the average chemical exchange rate; \(k_{obs}\) indicates the observed exchange rate (18)). The lower limit of \(\Delta G\) for the unfolding reaction was estimated according to 

\[
\Delta G = -RT \ln K_{unf} - RT \ln (k_{obs}/k_{ch})
\]

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RESULTS

Conformational Flexibility of DnaK—We initially measured deuterium incorporation into wild-type DnaK in the absence of nucleotides or substrates to analyze the overall kinetics of the HX reaction. We therefore incubated DnaK for different time intervals in D$_2$O, quenched the reaction by lowering the pH to 2.2 and the temperature to 0 °C, and subsequently analyzed the reaction on an HPLC-electrospray ionization tandem mass spectrometry setup (16, 19). Deuterons incorporated into full-length DnaK with apparent triple exponential kinetics leaving ∼40% of amide protons unexchanged after 1 h (data not shown; see Fig. 2). A protected core of 40–50% of all amide protons, which do not exchange under such conditions, is typical for a well folded native protein.

To localize fast and slow exchanging regions in DnaK and to elucidate the flexible parts in the SBD, which allow substrate binding in the high affinity state, we performed the analysis of the HX reaction on our HPLC-MS setup, including two columns with immobilized pepsin and A. saitoi protease XIII, both of which are active under quench conditions at 0 °C, to generate on-the-fly peptide fragments of DnaK. The average peptide size was around 17 residues, and the overall sequence coverage with peptides that could be detected in every run was about 80% (Fig. 1a). Sample spectra are shown in Fig. 1, b and c; secondary structure representations of the NBD and SBD of DnaK are shown color-coded according to the relative deuteron incorporation after 10 and 60 s and 10 and 60 min.

Overall there is good agreement between crystallographic data and the protection against HX. Well folded secondary structure elements incorporate deuterons to a lower degree than coil regions or less well defined helices and sheets. After 10 s of incubation in D$_2$O, only the C-terminal region of the SBD exchanged its amide protons almost completely for deuterons. This is not surprising because the corresponding peptide includes most of the 30 C-terminal residues not contained in the crystal structure and therefore not visible in Fig. 1c, which were shown by NMR to be unstructured (20). The outer loops (L$_{1,2}$ and L$_{5,6}$) of the β-sheet subdomain of the SBD exchange amide protons faster than the inner loops (L$_{1,2}$ and L$_{4,5}$), which are packed against helices aA and aB. Interestingly, helix aB incorporates deuterons surprisingly fast with 20 of the 32 amide protons (62%) exchanged after 10 min and 26 (81%) after 60 min, leaving only 12 and 6 amide protons protected corresponding to three and one and a half-helix turns, respectively. These data indicate significant flexibility of this helix with transient opening of hydrogen bonds.

The NBD subdomain IA appears to incorporate deuterons more rapidly than other parts of the NBD. However, because the resolution of our method is limited by the size of the peptides analyzed, close inspection of the data showed that most of the deuteron incorporation into subdomain IA can be accounted for by the unstructured coil regions. For example, the peptide, which includes the C-terminal helix of the NBD and the N-terminal part of the SBD, residues 371–391 (yellow in the 60-s time point in Fig. 1c), incorporates 11 deuterons within 60 s leaving 9 amide protons unexchanged. Even after 1 h, seven amide protons remain protected from exchange, corresponding to two helix turns, which is exactly the number of turns of the C-terminal helix of the NBD that is included in the peptide. It is therefore likely that this helix is very stable and that the part, which links the NBD to the SBD (indicated as dotted line in Fig. 1c), is almost completely solvent-accessible, consistent with the recent crystal structure of the two-domain construct of Hsc70 (11).

Nucleotide-dependent Solvent Accessibility Changes in Full-length DnaK—To assess the effects of nucleotides on the solvent accessibility of amide protons of DnaK, we first determined the overall kinetics of nucleotide-induced changes in HX. To analyze HX in DnaK in the presence of ATP over longer incubation times without interference with ATP hydrolysis, we used the DnaK-T199A mutant protein that is deficient in ATP hydrolysis but still proficient in ATP binding and undergoes ATP-induced conformational changes in the SBD like the wild-type protein (21). This protein was incubated in D$_2$O in the nucleotide-free or ATP-bound state and analyzed after different time intervals by our HPLC-MS setup excluding the columns with immobilized proteases. As for wild-type DnaK deuterium incorporation into DnaK-T199A occurred with apparent triple exponential kinetics ($\tau_1 = 2.5$ s; $\tau_2 = 110$ s; $\tau_3 = 1430$ s) leading to the exchange of 354 (58%) of a total of 613 amide protons after 60 min (Fig. 2a). HX in the ATP-bound state occurred with similar kinetics ($\tau_1 = 2.0$ s; $\tau_2 = 73$ s; $\tau_3 = 1670$ s) but, surprisingly, leveled off at a significantly lower number of total incorporated deuterons (327) as compared with the nucleotide-free state (Fig. 2a). Because biochemical evidence, including substrate association and dissociation measurements and partial tryptic digestion, clearly demonstrated that ATP binding induces an opening of the SBD, we hypothesized that ATP-induced increase in solvent accessibility in the SBD may be counteracted by a decrease in solvent accessibility in other parts of the protein. We therefore repeated the experiment adding ATP at a later time point (20 min, >10 $\tau_2$) when most of the rapidly exchanging amides in nucleotide-free DnaK were already deuterated. Under these conditions addition of ATP led to the incorporation of

6 When referring to peptides, the actual residue numbers are given starting the count with the initiator methionine, which is removed in vivo and is therefore not present in our DnaK preparations. When referring to exchanged amide protons, the first residue of the analyzed peptide is omitted because it carries an amino group instead of an amide group, which rapidly back-exchanges any incorporated deuterons during the desalting step.

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seven additional deuterons as compared with the control without addition of ATP (Fig. 2b). These data show that ATP binding to the NBD leads to an increased solvent accessibility in some parts of the proteins that is counterbalanced by a decreased solvent accessibility in other parts.

Localization of Nucleotide-induced Conformational Alterations—To localize the regions within DnaK with increased and decreased solvent accessibility, we repeated the analysis using the HPLC-MS setup with included protease columns (Fig. 3).

24 of 29 analyzed segments of DnaK incorporated less deuterons in the presence of ATP as compared with the nucleotide-free state consistent with our data on the full-length protein. The /H9252-sheet subdomain of the SBD (fragments 413–437 to 486–511) exchanged amide protons more rapidly in the ATP-bound state as compared with the nucleotide-free state. In contrast, helix /H9251 and the C-terminal part of the SBD exchanged amide protons more slowly (Fig. 3, a and b). Most segments of the NBD incorporated deuterons more slowly in the ATP-bound state as compared with the nucleotide-free state indicating a tightening of the NBD conformation (Fig. 3, a and b). Although the differences are small, they are highly reproducible and well reflected in the kinetics of deuteron incorporation as shown for selected segments in Fig. 3c. Most prominent is the difference between nucleotide-free and ATP-bound states in the linker region (Fig. 3, a and b, segments 372–391).

As comparison we analyzed the DnaK-R151A variant, which we recently identified as completely devoid in interdomain communication (22) (Fig. 3d). ATP-induced changes in deuteron incorporation into the NBD during a 1-min incubation in D2O were similar in this variant protein as compared with the wild-type protein (compare Fig. 3, a and d,
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FIGURE 4. Comparison of deuteron incorporation into wild-type DnaK and isolated NBD and SBD. a, difference of deuteron incorporation into DnaK-(2–638) minus deuteron incorporation into the NBD (DnaK-(2–385)) in the nucleotide-free state (white bars) or the presence of ATP (black bars) after 1 min of incubation in D2O. b, difference of deuteron incorporation into DnaK-(2–638) minus deuteron incorporation into the SBD (DnaK-(389–638)) in the nucleotide-free state (white bars) or the presence of ATP (black bars) after 1 min of incubation in D2O. c, secondary structure representation of the SBD (left) and the NBD (right) with specific segments in black and residue positions indicated. d, mass spectra of the peptic peptide residues 512–532 (representing the C-terminal part of helix a8 and the N-terminal part of zB; m/z = 821.74, y = 3) of wild-type DnaK in the nucleotide-free state (K–ATP) or the presence of ATP (K + ATP) and of the same peptide from the SBD after 1 min of incubation in D2O. The undeuterated peptide is shown for comparison at the bottom. The spectra were obtained using specific conditions for eluting the peptides from the reversed-phase column. Spectra for the fully deuterated peptide could not be obtained.

The comparison of the deuteron incorporation into the isolated SBD (DnaK-(389–638)) with the incorporation into full-length DnaK also revealed surprising results (Fig. 4b). Overall, the isolated SBD exchanged in five of the eight segments investigated more amide protons than the full-length protein in the nucleotide-free state (Fig. 4b, white bars). This is most pronounced in segment 486–511, comprising half of z-strand 7, z-strand 8, and the first turn of helix aA, which exchanges on average 6.6 of the 26 amide protons in the full-length protein but 13.9 amide protons in the isolated SBD.

Because helix aA of the SBD contains the major contact sites with the NBD in the recent crystal structure of the two-domain construct of Hsc70, we were eager to see whether these contacts would be measurable in solvent accessibility of this helix. By using an altered solvent gradient of the reversed-phase column, we were able to analyze a peptic peptide that contained the C-terminal part of helix aA and the N-terminal half of helix aB (Fig. 4d, 512–532). This peptide showed one deuteron difference when incubating the isolated SBD as compared with full-length DnaK in the nucleotide-free state suggesting only a slight stabilizing influence of the NBD on this region.

When the isolated SBD is compared with full-length DnaK in the ATP-bound state, significant differences are still observed (Fig. 4b, black bars). The four C-terminal segments of the SBD still incorporate less deuterons in the full-length protein as compared with the isolated SBD, and only two segments (413–437 and 439–457) exchanged more in the full-length protein demonstrating the ATP-induced opening of the substrate-binding pocket. Similarly, the region 512–532 incorporates at least four deuterons more in the full-length protein in the presence of ATP than in the isolated domain, indicating that ATP-induced conformational changes also lead to an increased solvent accessibility of the amides of helices aA and aB (Fig. 4d). Taken together our comparison of HX into the isolated domains of DnaK with HX of full-length DnaK segments 3–15 to 358–370). However, ATP-induced changes in deuteron incorporation into the SBD were strongly reduced (compare Fig. 3, a and d, segments 401–408, 413–437, 486–511, and 602–628). Most notable was the difference in segment 372–391. Upon ATP binding, six amide protons were protected from exchange in the wild-type protein, but only 1 was protected in the DnaK-R151A variant.

Mutual Influence of the Two Domains on Each Other—To analyze the mutual influence of the two domains on each other, we compared deuteron incorporation of the isolated domains with the incorporation into full-length protein in the nucleotide-free state and in the presence of ATP. Fig. 4a shows the difference plot of deuteron incorporation into wild-type DnaK (D_{DnaK-(2–638)}) minus incorporation into the NBD (D_{DnaK-(2–385)}). Most segments of the NBD incorporated a similar number of deuterons when the full-length protein was incubated for 1 min in D2O as when the isolated NBD was incubated. For some segments scattered throughout the NBD, deuterons were incorporated more rapidly in the isolated domain as compared with the full-length protein, indicating a slightly increased flexibility of the isolated domain.

In the presence of ATP, four additional segments (segments 139–163, 164–177, 338–356, and 372–382) exhibit slower deuteron incorporation in the full-length protein as compared with the isolated NBD (Fig. 4, a and c). These four segments represent two regions of the NBD, the subdomain IA and parts of subdomains IIA and IIB that are located on the opposite face of the NBD as compared with the region in subdomain IA.

In subdomain IA enclosing loops in the SBD. If communication between the two domains is compromised by a mutational replacement in subdomain IA, the changes in the SBD are no longer observed. HX analysis thus allows us to monitor the conformational changes occurring in DnaK during ATP-dependent interdomain communication.

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indicates that the two domains stabilize each other, an effect that is much more pronounced in the SBD than in the NBD.

Substrate-induced Conformational Alterations—To analyze possible substrate-induced conformational changes in the SBD and the NBD, we compared the HX of full-length DnaK in the absence and presence of the peptide α32-Met195–Asn207, which was identified earlier to be a high affinity substrate for DnaK (15), and in the absence and presence of ATP. In the absence of ATP, overall little changes in deuteron incorporation were observed except for the segments that form the substrate-enclosing loops L1,2 and L3,4. The HX kinetics of these two segments are shown in Fig. 5. The segment representing the inner loop L1,2 (residues 401–408) incorporates one to two deuterons very rapidly and a third over a period of about 1 h in the absence of the substrate peptide, whereas in the presence one of the three amide protons is completely protected from exchange (Fig. 5a). This result is consistent with the crystal structure that shows one amide proton completely free, one in a loose internal hydrogen bond (distance between amid N and carbonyl O 3.4 Å), and one forming a hydrogen bond to the backbone of the substrate peptide (4). The HX kinetics of the segment representing the outer substrate enclosing loop L3,4 (residues 413–437) changed in a more surprising way upon substrate binding. In the absence of the substrate peptide, about six deuterons were incorporated within the shortest time interval measured (10 s). Consistent with the x-ray structure, 6 of the 24 amide protons directly coordinated to water (Protein Data Bank entry code 1DKX). Deuteron incorporation continued more slowly to arrive at 15 after 1 h, leaving 9 amide protons protected and consistent with the number of strong hydrogen bonds (O-N distance below 3 Å) within this segment in the x-ray structure. In the presence of a substrate deuteron incorporation was much slower. Only three amide protons exchanged within the first 10 s, and after 1 h deuteron incorporation approached 10 leaving 14 amide protons completely protected within this time frame. Because only two hydrogen bonds are formed with the substrate in the crystal structure, it must be concluded that parts of the segment represented by the analyzed peptide are stabilized upon substrate binding.

To analyze the allosteric mechanism by which substrates induce ATP hydrolysis, we wanted to measure HX in the presence of peptide and ATP. To circumvent the problem of ATP hydrolysis during the incubation in D2O, we made use of the DnaK-T199A variant. None of the resulting mass spectra showed indications for a bimodal distribution of the isotopic peaks, indicating that all DnaK molecules were in the same conformation. Fig. 5c shows the comparison of deuteron incorporation after 1 min in D2O into selected segments of nucleotide-free DnaK-T199A in the absence or presence of the peptide α32-Met195–Asn207 and in the absence or presence of ATP. Three different situations can be distinguished. First, the effects of ATP are dominant over the effects of peptide (segments 17–41 and 401–408) indicating that ATP was not hydrolyzed during the incubation time. Second, the effects of peptide are dominant over the effects of ATP (segments 139–163, 165–177, and 372–391) suggesting conformational changes that could be linked to peptide α32-Met195–Asn207 (MAPVLYLQDKSSN (15)). Insets show same data with a logarithmic time scale. c, ATP- and substrate-induced conformational changes. Nucleotide-free DnaK-T199A was incubated in the absence (white and hatched bars) and presence (gray and black bars) of ATP and absence (white and gray bars) and presence (hatched and black bars) of peptide α32-Met195–Asn207 for 1 min in D2O. Shown is the deuteron incorporation into selected segments as indicated. Numbers in parentheses give the number of exchangeable amide hydrogens in the segments. d, deuteron incorporation into the linker segment 372–391 after 1 min and 3 min in D2O in the absence or presence of ATP or peptide. pep→ATP indicates incubation of nucleotide-free DnaK-T199A in the presence of peptide for 1 min in D2O and then addition of ATP and incubation for additional 2 min. ATP→pep indicates incubation of ATP-bound DnaK-T199A for 1 min in D2O and then addition of peptide and incubation for an additional 2 min.
allostERIC conTrol (discussed in detail below). Third, the effects of ATP and peptide seem independent and additive (segment 413–437) suggesting that this segment consists of parts that react independently one according to the first situation and one according to the second situation. To analyze the ATP and peptide effects more closely, we performed order-of-addition experiments. We incubated nucleotide-free DnaK-T199A in the presence or absence of ATP or peptide for 1 min in D₂O, then added peptide to the ATP-containing sample or ATP to the peptide-containing sample, and continued incubation for an additional 2 min and compared the deuteron incubation with samples to which nothing was added. The outcome was principally the same as in the previous experiment and exemplarily shown for the linker segment in Fig. 5d. Peptide overrides the effect of ATP on this segment of DnaK and leads to an exposure of the linker to solvent. Taken together these data clearly indicate that ATP-induced conformational changes in some parts of the NBD and the linker are reversed by peptide binding suggesting a mechanism for the mutual allosteric control of the two domains.

**DISCUSSION**

This study revealed several new insights into the structural dynamics of Hsp70 proteins and the ATP-dependent communication between the nucleotide- and substrate-binding domains, which mediates the allosteric regulation of this class of chaperones. Nucleotide-dependent changes in deuteron incorporation demonstrate an overall tighter folding of the NBD and an increased solvent exposure of several regions in the SBD visualizing the conformational changes accompanying the opening of the substrate-binding pocket. Comparison of the isolated domains with the full-length protein indicates a stabilizing effect of the domains onto each other, which is specifically pronounced in the lower β-sheet of the substrate-binding domain. The observation that the linker is completely accessible to solvent in the absence of nucleotides and solvent protected in the presence of ATP reveals nucleotide-dependent conformational changes of the linker, which may be part of the mechanisms by which the two domains communicate with each other.

The time-dependent incorporation of deuterons into DnaK in the nucleotide-free state allows three conclusions. First, the NBD shows significant flexibility consistent with a shearing movement of the four subdomains relative to each other that was recently proposed based on significant flexibility consistent with a shearing movement of the four nucleotide-free state allows three conclusions. First, the NBD shows other.

Second, the lid forming helix αB in the SBD exchanges much more rapidly than expected for a well folded helix, leaving the hydrogen bonds of only 3 and 1.5 helix turns intact over a period of 10 and 60 min, respectively. This is consistent with local unfolding that accompanies the opening movement, which allows substrate binding and release even in the absence of ATP. Such a movement was proposed by Hendrickson and co-workers (4) on the basis of a second crystal form in which helix αB was bent upward by 11°. This flexibility of helix αB would not be necessary in other models, which propose that the opening of the lid occurs either by a pivoting movement of the entire lid around helix αA or by an upward movement of the entire helix αB without helix melting and with a hinge between helix αA and αB (23, 24). Our data are also consistent with the NMR and x-ray structures of truncated versions of the SBD of DnaK and Hsc70, which show a helical secondary structure only up to residue 535 (11, 24, 25).

The estimated half-life of the NBD in the nucleotide-free state is similar to the half-life of the DnaK-peptide complex (~10 min). Consistently, the substrate enclosing outer loops L3,4 and L5,6 and β-strand 3 incorporate deuterons with similar kinetics. Third, the C-terminal part of the NBD and the linker that connects NBD and SBD are highly solvent-exposed in the nucleotide-free state, in agreement with data from partial tryptic digestion, which shows an exposed cleavage site N-terminal of the linker, and the recent crystal structure of a two-domain construct of bovine Hsc70 (11, 26). Taken together, Hsp70 proteins show a high degree of flexibility in both NBD and SBD.

The comparison of deuteron incorporation into full-length DnaK with the incorporation into DnaK-(2–385) in the absence of nucleotide revealed small changes throughout the NBD. These data are consistent with a recent NMR study, which compared chemical shifts of the residues in the NBD in a T. thermophilus DnaK two-domain construct with the isolated NBD (27). It was found that chemical shift perturbations caused by the presence of the SBD are scattered throughout the NBD. The absence of a defined area, which is protected by the presence of the SBD, is surprising. It should be noted, however, that mere side chain interactions, which do not affect the backbone amide hydrogen accessibility, would not be detected by this method. The differences in HX observed in the presence of ATP as compared with the nucleotide-free state revealed three regions of the NBD, the major part of subdomain IA and small parts of subdomains IB and IIA, both of which are located on the opposite face of the NBD as compared with the region in subdomain IA. The slight stabilizing effect exerted by the SBD on the NBD is therefore enhanced by ATP binding to the NBD, indicating a more tightly coupled state. These data provide a structural basis for earlier observations with small x-ray scattering and partial tryptic proteolysis (26, 28).

Both investigations show that the NBD and SBD are more tightly coupled with each other in the ATP-bound state as compared with the nucleotide-free state with a smaller radius of gyration of the full-length protein and the linker connecting both domains not accessible for proteolytic cleavage.

The deuteron incorporation kinetics for full-length DnaK exhibited tri-exponential characteristics indicating different degrees of protection for individual groups of amide hydrogens. Comparison with the theoretical average chemical exchange rate for DnaK as calculated using the HXPe program (courtesy Z. Zhang; see “Experimental Procedures”) allowed the determination of the global protection factors. These protection factors ranged from 30 for the fast exchanging amide hydrogens to over 17,000 for the slow exchanging hydrogens in the absence of nucleotide and from 25 to over 20,000 in the presence of ATP. Because the protection factors are proportional to the stabilization energy of the protein, the difference in global stabilization energy ΔΔG between the nucleotide-free and the ATP-bound states can be estimated to at least 160 kJ mol⁻¹. This global stabilization of DnaK by ATP binding is composed of a more prominent stabilization in the NBD and a destabilization in the SBD as demonstrated by the HX difference map shown in Fig. 3a.

The comparison of amide hydrogen exchange of full-length DnaK in the presence and absence of ATP, as well as with the isolated NBD at the peptide level, provides insights into the allosteric regulatory mechanism by which ATP binding induces the opening of the substrate-binding pocket and by which substrate binding stimulates the ATPase activity. In the NBD the segments with the largest nucleotide-dependent changes in HX kinetics of full-length DnaK cluster in subdomain IA (Fig. 3, b and c), as do the changes between full-length DnaK and DnaK-(2–385) in the presence of ATP (Fig. 4, a and c). The nucleotide-dependent alterations of HX are explained easily for the segments 3–15 and 165–177. The former contains residues Asp8, Thr11, Thr12, Tyr13, and Cys15 corresponding to Asp10, Thr13, Thr14, Tyr15, and Cys17 of bovine Hsc70, which contact the β- and γ-phosphate of the bound ATP in the crystal structure, and nucleotide binding may therefore stabilize this loop (6, 29). The latter segment (165–177) contains Glu171 corresponding to Glu175 in Hsc70 that is involved in the coordination of Mg²⁺ and
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may therefore also be stabilized by ATP binding (7). The segments 139–163 and 372–391 do not have such an obvious connection to nucleotide binding. The first region, however, contains Pro143 that was recently shown to constitute the molecular switch, which stabilizes the two alternating conformations of Hsp70 proteins, and Arg151, which relays the proline switch position and therefore ATP binding to the SBD and substrate and DnaJ binding to the NBD (22). The second region contains the linker that connects NBD and SBD. Alterations in the HX behavior in this region are particularly interesting because it also has implications on the allostERIC interdomain control mechanism. In the peptic digest we found one smaller peptide that only contains residues 389–392, which exchanges all exchangeable amide protons within 10 s in the nucleotide-free state but is almost completely protected in the ATP-bound state (Fig. 3b). In the nucleotide-free state this segment therefore is solvent-accessible and presumably is an unstructured loop. Upon ATP binding, this segment either forms secondary structure or hydrogen bonds to parts of SBD or NBD. Because the residues in this region are highly conserved, we propose that these conformational changes are intimately linked to allostERIC interdomain control. Consistent with this hypothesis are the earlier observations that replacement of the hydrophobic residues 389–392 with alanine (30) completely blocks changes are intimately linked to allosteric interdomain control. Consist-
ent with this hypothesis are the earlier observations that replacement of the hydrophobic residues 389–392 with alanine (30) completely blocks ATP-induced stimulation of substrate release and substrate and DnaJ-mediated triggering of ATP hydrolysis. The linker, which is exposed in the nucleotide-free state and becomes protected upon ATP bind-
ing, therefore seems responsible for triggering the conformational change in the NBD that is necessary for the induction of ATP hydroly-
ysis. Consistent with this hypothesis is the fact that an NBD con-
struct, including the linker (DnaK-(2–393)), has a strongly increased ATPase activity similar to full-length DnaK in the presence of DnaJ and a substrate protein.7

When the mutual allostERIC control between the NBD and the SBD is abolished by the Arg151 to Ala replacement (22), most nucleotide-de-
dependent changes in the HX kinetics in the NBD are still observed but not the changes in the linker or the SBD (Fig. 3d). Together these data clearly demonstrate that the HX kinetics reveal segments in Hsp70 proteins that are involved in nucleotide-dependent allostERIC control. In particular the linker region seems to play a pivotal role in this mechanism.

Our comparison of HX into the isolated SBD with the HX of full-
length DnaK indicated a strong stabilizing effect of the NBD on the SBD, which may be part of the linker-mediated allostERIC control. The largest difference in deuteron incorporation between full-length DnaK and the SBD was observed in segment 486–511 (Fig. 4, b and d). In the isolated SBD we observed an exchange of around 14 of the 26 amide protons within a time span of 1 min demonstrating an unexpectedly high degree of flexibility in this region. The protection of more than six amide protons observed by us for the full-length protein indicates that the NBD stabilizes this region significantly. The recent crystal structure of an Hsp70 protein in the nucleotide-free state that contains the NBD and parts of the SBD shows the helix αA of the SBD docked onto subdomain IA of the NBD (Protein Data Bank code entry 1YUM (11)). Because in this structure no additional hydrogen bonds are observed between the NBD and region 486–511, the stabilizing effect must be transduced through helix αA and the entire β-sheet.

The ATP-induced changes in deuteron incorporation into the SBD lead to increased HX in four segments (residues 413–437, 439–457, 459–473, and 486–511) representing the β-sheet subdomain and a decreased HX in helix αB and the C-terminus (Fig. 3, a and c and Fig. 4d). This demonstrates that the ATP-induced opening of the SBD involves mainly the β-sheet subdomain and not helix αB consistent with earlier biochemical data (31) but incompatible with a mechanism that pro-
poses melting of helix αB as the first step for SBD opening (4). The increases in deuteron incorporation into β-sheet 3 and the outer sub-
strate enclosing loops L1,4 (residues 413–437) and L5,6 (residues 458–473) are obvious requirements for the opening of the substrate-binding pocket. The increase in HX kinetics of the inner loop L4,5 (residues 439–457) is surprising because the inner loops together with the framing helices αA and αB appear to constitute a stable entity in the crystal structure. However, Hendrickson and co-workers (4) pointed out the unexpected high number of charged residues in this region. The increased deuteron incorporation may be linked to the interdomain communication and the mechanism through which ATP effects the opening of the SBD. Interestingly, residue Lys414, which is essential for interdomain communication, is close to the base of this loop (32). In addition, segment 512–532, comprising the C-terminal part of helix αA and the proximal half of helix αB, incorporates at least four deuterons more in the ATP-bound state as compared with the nucleotide-free state. There are two alternative explanations for this observation. Either the proximal part of helix αB or the C-terminal part of helix αA becomes more flexible. In the first case this would indicate a local unfolding that accompanies the opening of the lid, which together with the absence of large changes in solvent accessibility of the distal half of helix αB suggests the proximal part of the helix as hinge region for the ATP-induced lid opening; this scenario is consistent with the increased solvent accessibility of the inner loop L4,5. In the second case this could indicate a detachment of the upper part of helix αA from the NBD as part of the ATP-induced movement of both domains relative to each other, which would lead to a burial of the linker region consistent with the loss of solvent accessibility of the linker. In consideration of all available data, it seems that a combination of both models is most likely.

Substrate binding to nucleotide-free DnaK reduced solvent accessibility in two segments of the SBD by one and six amide hydrogens, respectively. While the protection observed in segment 401–408 comprising parts of β-sheets 1 and 2 and loop L1,2 corresponds exactly to the hydrogen bond between this part of the SBD and the substrate peptide suggested by the x-ray structure, the protection in segment 413–437 comprising the loop between β-sheets 2 and 3, β-sheet 3, and loop L1,4 exceeds the expected reduction of solvent accessibility through hydro-
gen bonds with the substrate peptide by four amide hydrogens. Although an exact localization of the stabilized position is not possible because of the low spatial resolution of our MS analysis in this part of the SBD, it is likely that conformational changes in the loop between β-sheets 2 and 3 contribute at least in part to this loss of solvent acces-
sibility, because this part contains Lys414 implicated in the interdomain communication (32). This hypothesis is supported by the results in the presence of ATP (Fig. 5c) showing the protection of exactly two amide hydrogens after 1 min in D2O presumably corresponding to the two hydrogen bonds formed with the peptide substrate, whereas the overall deuteron incorporation increased.

The ATP-induced changes in solvent accessibility were affected or even totally reversed by the peptide substrate in four segments (139–
163, 165–177, 372–391, and 413–437). These results have two impor-
tant implications. First, they show the protein segments involved in transmitting the substrate-binding signal to the catalytic center. Sec-
ond, they suggest that substrate triggers ATP hydrolysis by reverting certain ATP-induced conformational changes and converting certain parts of the protein into an ADP-like state. These results are consistent but go well beyond earlier observations that peptide binding leads to an

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increase of intrinsic tryptophan fluorescence that precedes ATP hydrolysis (33). Especially interesting is the substrate-triggered reversion of the ATP-induced conformational change in segment 139–163, which is fully consistent with the recently proposed proline switch (22). We showed in that publication that Pro\textsuperscript{143} in the NBD undergoes a conformational change, possibly a trans to cis isomerization, upon ATP binding. This conformational change is relayed to the SBD through Arg\textsuperscript{151}. Substrate was proposed to trigger ATP hydrolysis by inducing the reversion, cis to trans, of this conformational change. The changes observed in segment 165–177 most likely also contribute to the stimulation of ATP hydrolysis because the Mg\textsuperscript{2+}-coordinating Glu\textsuperscript{171} is in this segment. The two other segments with significant effects of peptide on the ATP-induced conformational change seem to mark the pathway by which peptide triggers ATP hydrolysis.

Taken together our data suggest the following mechanism of allosteric interdomain control. ATP binding to the NBD induces a conformational change in segment 139–163 and 165–177 most likely in Pro\textsuperscript{143} (trans-cis isomerization), Arg\textsuperscript{151}, and Glu\textsuperscript{171}. This leads to a conformational change in the linker region (segment 372–391), which becomes protected from solvent and is transduced from there to segment 413–437 most likely onto the loop between β-sheets 2 and 3 of the SBD around Lys\textsuperscript{414}, resulting in an opening of the substrate-binding pocket with increased solvent accessibility of segment 439–457. Substrate binding reverts all of these conformational changes, in particular in Pro\textsuperscript{143} (cis-trans isomerization) and Glu\textsuperscript{171}, thereby bringing the catalytic Lys\textsuperscript{46} with its coordinated water molecule into the ideal position for γ-phosphate cleavage.

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