Direct Comparison of a Stable Isolated Hsp70 Substrate-binding Domain in the Empty and Substrate-bound States**

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The Hsp70 family of molecular chaperones acts to prevent protein misfolding, import proteins into organelles, unravel protein aggregates, and enhance cell survival under stress conditions. These activities are all mediated by recognition of diverse hydrophobic sequences via a C-terminal substrate-binding domain. ATP-binding/hydrolysis by the N-terminal ATPase domain regulates the interconversion of the substrate-binding domain between low and high affinity conformations. The empty state of the substrate-binding domain has been difficult to study because of its propensity to bind nearly any available protein chain, even if only modestly hydrophobic. We have generated a new stable construct of the substrate-binding domain from the Escherichia coli Hsp70, DnaK, which has enabled us to compare the empty and peptide-bound conformations using NMR chemical shift analysis and hydrogen-deuterium exchange. We have determined that the empty state is, overall, quite similar to the peptide-bound state, contrary to a previous report. Peptide binding leads to a subtle alteration in the packing of the α-helical lid relative to the β-subdomain. Significantly, we have shown that the chemical shifts of the substrate-binding domain and the ATPase domain do not change when they are placed together in a two-domain construct, whether or not peptide is bound, suggesting that, in the absence of nucleotide, the two domains of E. coli DnaK do not interact. We conclude that the isolated substrate-binding domain exists in a stable high affinity state in the absence of influence from a nucleotide-bound ATPase domain.

Escherichia coli DnaK is a member of the Hsp70 family of molecular chaperones, which facilitate folding of nascent protein chains, enhance recovery after cellular stresses such as heat shock, aid in protein translocation across membranes, and participate in protein disaggregation and degradation (1). In addition to these very general functions, which rely on ATP-regulated binding to short hydrophobic stretches of substrate proteins, Hsp70s also play specific cellular roles in disrupting macromolecular complexes such as clathrin coats. When ADP is bound to the 45-kDa N-terminal ATPase domain, the 25-kDa C-terminal substrate-binding domain (SBD) binds substrates with high affinity and slow on/off kinetics. However, ATP binding converts the SBD to a low affinity conformation characterized by rapid substrate on/off rates and enhanced proteolytic susceptibility (2, 3). Small angle x-ray scattering data collected on the related protein Hsc70 (4) implicates a more intimate association between the two domains in the ATP-bound conformation, and in this state, the sole intrinsic tryptophan of DnaK, Trp-102, is buried at the interface with the SBD (3, 5). Not only does nucleotide binding influence the peptide-binding pocket, but peptide binding also speeds the ATP hydrolysis rate, which is the rate-limiting step of the cycle for E. coli DnaK. The lack of a high resolution structure including both domains has hampered our understanding of the mechanism of allostery between these two binding sites. Nevertheless, increasing evidence of a role for dynamics suggests that even high resolution structures will be only the beginning of the story (6, 7).

The Hsp70 ATPase domain shares structural similarity with actin and binds nucleotide in a deep cleft between two large lobes (8, 9). A short well conserved linker connects the ATPase domain with the SBD, which itself consists of a β-subdomain capped by an α-helical lid subdomain, as observed in an x-ray crystal structure of DnaK-(389–607) bound to a peptide (Fig. 1A) (10). The substrate peptide NR (sequence NRLLLTTG) is nestled between loops of the distorted β-sandwich in an extended conformation (11) and is encapsulated by the α-helical lid subdomain, which extends over the top of the peptide-binding pocket, but does not directly contact the peptide. Based on observation of a kink in the α-helical lid in one crystalline form of the peptide-bound SBD, it was proposed that ATP binding could cause the α-helical lid to move away from the β-subdomain (10). However, it is now clear that, although such a conformational change in the helix may indeed occur, the α-helical lid itself is dispensable for the ATP-induced switch of the β-subdomain to the low affinity conformation (3, 5, 6, 12–14), and therefore more extensive structural changes must occur throughout the β-subdomain.

Insight into this change in conformation plus the role of peptide in allostery should be aided by structures of the SBD with and without bound substrate. However, structures of the empty conformation of the SBD have been difficult to obtain because of the strong tendency of the domain to bind any available hydrophobic sequence. In NMR structures of truncated SBDs of DnaK and Hsc70, in which three C-terminal helices were removed (corresponding to DnaK residues 386–561 and Hsc70-(383–540)) (15, 16), leucine residues near the new C terminus bound intramolecularly into the peptide-binding pocket. A subsequent structure of the isolated β-subdomain (DnaK residues 393–507) in the empty conformation revealed a highly flexible structure with a major conformational change in strand β3 at the edge of one sheet (14). The dynamic nature of the empty β-subdomain and its low affinity for NR peptide (Kd value of 0.6 mM) indicated that the isolated subdomain might favor the low affinity conformation. However, this fragment was later reported to exist as a dimer in the empty conformation, and thus it is not clear at this time which conformational changes might be due to dimer formation and which due to loss of peptide (17).
Here we demonstrate that, contrary to the reported behavior of the isolated β-subdomain, a DnaK SBD fragment retaining a portion of the α-helical subdomain but harboring mutations that abrogate self-bind-
ing was well folded and stable in the empty state and bound peptides with high affinity. The structural and dynamic changes in the empty state relative to the peptide-bound form were small and local and reported on conformational rearrangement of the peptide-binding pocket and repacking of the helix against the β-subdomain. When this stable SBD and the native ATPase domain were linked in a two-domain construct, the protein was allosterically functional. Strikingly, however, NMR spectra of the two-domain construct without nucleotide present indicated that the two domains do not interact, even in the presence of peptide. These results have demonstrated that nucleotide is necessary to establish allosteric signaling between the domains.

EXPERIMENTAL PROCEDURES

Cloning Strategies—Plasmid pRLM212 was a kind gift from R. McMacken (Johns Hopkins University), and codes for H4-DnaK-(387–552), under control of a heat shock promoter. The L542Y/L543E mutant, H4-DnaK-(387–552)-ye, was created by QuikChange PCR on pRLM212. Plasmid pMS-DnaK-(1–552) was created from the wild-type DnaK expression vector pMS-DnaK (18) by QuikChange PCR mutagenesis of codons 553 and 554 to stop codons. This plasmid was then further mutagenized to create the L542Y/L543E version by QuikChange PCR as above for pRLM212. All coding sequences were verified by full DNA sequencing (Davis Sequencing, Davis, CA). pRLM157, which encodes DnaK-(1–388) under a heat-inducible promoter, was also provided by Dr. Roger McMacken.

Protein Purification—Constructs containing the ATPase domain were transfomed into the DnaK-deficient BB1553 strain of E. coli, grown at 30 °C, and induced either by the addition of 0.4 mM isopropyl 1-thio-β-D-galactopyranoside or heat shock at 42 °C, depending on the promoter, and purified as described previously (18) with a minor modification. In the ATP-agarose used in this study, the N6 atom of ATP was linked to agarose instead of the C8 atom (Sigma catalog number A9264). DnaK can be eluted from this column using EDTA instead of excess ATP, resulting in completely nucleotide-free protein. Briefly, soluble protein extracts were loaded on DEAE-Sepharose equilibrated in 20 mM HEPES, 0.1 mM EDTA, pH 7.4, and eluted using a continuous gradient to 500 mM KCl. Peak fractions were pooled, brought to 10 mM MgCl2, and then loaded on the ATP-agarose column pre-equilibrated in 20 mM HEPES, 5 mM MgCl2, and 100 mM KCl, pH 7.6 (HMK buffer). The column was washed with HMK plus 2 mM KCl and the protein was eluted in 20 mM HEPES, 10 mM EDTA, 100 mM KCl, pH 7.6. Pooled peak fractions were concentrated in an Amicon ultrafiltration cell (Millipore, Billerica, MA), dialyzed against HMK buffer, and stored at −80 °C. The 260/280 nm absorption ratio of protein prepared in this way was always <0.6, indicating that the protein is nucleotide-free (19).

His-tagged SBD constructs were expressed in the BL21(DE3) strain of E. coli grown at 30 °C to an A600 of 1 and then induced at 42 °C for 5 h. Soluble extracts prepared by lysozyme treatment, sonication, and removal of cell debris by centrifugation were applied to a Ni2+–nitrilotriacetic acid resin (Qiagen, Valencia, CA) pre-equilibrated in 50 mM sodium phosphate, 0.5 mM NaCl, pH 8 (buffer A), and eluted using a gradient to 200 mM imidazole in buffer A. Peak fractions were pooled, concentrated in an Amicon ultrafiltration cell, and dialyzed against 20 mM sodium phosphate, 25 mM NaCl, pH 7 (buffer B). For H4-DnaK-(387–552)-ye, roughly half of the protein is expressed in inclusion bodies; therefore in this case, protein was also purified from the pellet by solubilizing in 8 M urea in buffer A, after which it was sonicated, centrifu-
ged, and chromatographed on Ni2+–nitrilotriacetic acid resin as above but with 8 M urea incorporated in all buffers. Peak fractions were concentrated by ultrafiltration, refolded by dropwise dilution into a 20-fold excess of buffer B with rapid stirring, concentrated again, and dialyzed against buffer B before storage at −80 °C. Protein purified from the soluble fraction and the refolded protein gave identical TROSY NMR spectra.

Preparation of NMR Samples—Proteins were labeled with 15N or 13C by growth in M9 minimal medium supplemented with [15N]ammonium chloride and [13C]glucose. Purified and concentrated protein was buffer-exchanged into a Centricron-10 concentrator (Millipore, Billerica, MA) into NMR buffers as follows: for SBD-only constructs, 10 mM sodium phosphate, 10 mM sodium acetate, 10% D2O and 0.02% sodium azide; for ATPase-domain-containing constructs, 10 mM potassium phosphate, 10 mM potassium chloride, 5 mM MgCl2, 5 mM d(β)-mercaptoethanol, 10% D2O and 0.02% sodium azide. All NMR buffers were pH 7 and contained 0.5 mM 3-(trimethylsilyl) propionate sulfonic acid as an internal standard.

NMR Spectroscopy—Assignments were determined by homonuclear and heteronuclear NOE detection (18). NMR spectra were analyzed with the software package NMRPipe (7) and visualized with NMRView (8). The chemical shifts (δ) were referenced to DSS (dimethylsulfoxide-d6) at δ 2.5 ppm. Assignments were confirmed by chemical shift correlations obtained from 1H,15N-NOESY/TROSY and 1H,15N-TOCSY/TROSY experiments collected on 15N-only samples (80- and 50-ms mixing times, respectively) and used to complete the assignments (20, 21). The programs Felix 2000 (Accelrys, Inc, San Diego, CA) and Xeasys (22) were used for spectral processing and data analysis, respectively. 98% of non-proline backbone resonances were assigned in this manner. Weighted average chemical shift changes (Δδavg) were calculated as described in Ref. 14.

NMR Hydrogen Exchange—Hydrogen-deuterium exchange was initiated by resuspending samples of 0.3 mM [15N]H4-DnaK-(387–552)-ye that had been lyophilized with and without 0.7 mM NR peptide on a Bruker Avance 600 spectrometer. A combination of HNCA, HN(CO)CA, CBCA(CO)NH, HNCA,CB, HNCO, and HN(CA)CO experiments using 13C/[15N]-labeled protein and 1H,15N-NOESY/TROSY and 1H,15N-TOCSY/TROSY experiments collected on 15N-only samples (80- and 50-ms mixing times, respectively) were used to complete the assignments (20, 21). The programs Felix 2000 (Accelrys, Inc, San Diego, CA) and Xeasys (22) were used for spectral processing and data analysis, respectively. 98% of non-proline backbone resonances were assigned in this manner. Weighted average chemical shift changes (Δδavg) were calculated as described in Ref. 14.

ATPase Assays—Single turnover ATPase rates were measured by following the recovery of the fluorescence intensity of Trp-102 after the addition of substoichiometric amounts of ATP (19, 24), using 1 μM protein and 0.5 μM ATP at 25 °C in HMK buffer. Fluorescence measurements were made on an Alpha Scan Fluorometer (Photon Technology International, Birmingham, NJ) with excitation at 295 nm, emission at 340 nm, and excitation and emission slits set at 2 and 5 nm, respectively. Shutteres were gated closed between data points to avoid photo-bleaching over the course of the experiment. Recovery curves were fit to a single exponential. Peptide-stimulated rates were measured by adding ATP after a 15 s preincubation of protein with 30 μM p5 peptide.

Fluorescence Measurements—The ATP-induced blue shift of Trp-102 was monitored with emission wavelength scans from 305–405 nm upon excitation at 295 nm (25 °C). Excitation slits were 2 nm and emission slits were 5 nm. Protein concentration was 1 μM in HMK buffer, ± 0.5 mM ATP.

λ Phage Replication Assays—These assays followed established protocols (18).
were deleted, corresponding to H6DnaK-(387–552), which was generated as a truncated domain in which the last three helices of the NMR. Because the full-length SBD is aggregation-prone, we began with binding the empty and the peptide-bound states of the DnaK SBD using {\textit{Comparison of basal and peptide-stimulated ATPase rates for truncated proteins relative to full-length DnaK. Single turnover ATPase rates for the indicated constructs were measured in the absence (hatched bars) and presence (filled bars) of 30 \mu M p5 peptide. Error bars represent standard deviations from two (DnaK-(1–552)) or three measurements (wild-type DnaK and DnaK-(1–552)-ye).}}

RESULTS

Design of a Truncated SBD Construct That Retained Full Substrate Binding—We set out to determine the conformational differences between the empty and the peptide-bound states of the DnaK SBD using NMR. Because the full-length SBD is aggregation-prone, we began with a truncated domain in which the last three helices of the \( \alpha \)-helical lid were deleted, corresponding to H6DnaK-(387–552), which was generously provided by Dr. Roger McMacken (Johns Hopkins University) (Fig. 1B). However, peptide titration monitored by NMR demonstrated that this fragment had extremely low affinity for substrates (data not shown) and had nearly identical chemical shifts to a slightly less truncated version of the DnaK SBD, where C-terminal tail residues occupied the substrate-binding pocket intramolecularly (16, 25). Interestingly, when this truncated domain was connected to the ATPase domain in cis (corresponding to DnaK-(1–552), Fig. 1B), the single turnover ATPase rate was 10-fold higher than that of full-length DnaK, and it was not stimulated in the presence of 30 \mu M p5 peptide (Fig. 2), consistent with the interpretation that the C terminus of the SBD fragment was occupying the binding pocket in an entropically favored interaction.

In the NMR structure of DnaK-(386–561) by Wang et al. (16), two adjacent leucine residues at positions 542 and 543 were bound intramolecularly by the substrate-binding pocket, presumably due to deletion of the \( \alpha \)-helical bundle into which these leucines normally pack (Fig. 1A) (10). In previous work, we destabilized the binding of the C-terminal tail by shifting to lower pH, presumably causing protonation of the histidines that flank these two leucine residues, but we were only able to populate the unbound conformation to a small degree (10–20\%) (25). Therefore, in the present work, we mutated these residues to tyrosine and glutamate (L542Y/L543E) to fully destabilize this apparently non-physiological interaction with the binding site and to observe the empty conformation of the SBD.

The resulting SBD fragment, H6DnaK-(387–552)-ye, had restored high affinity peptide binding when monitored using either NMR (Fig. 3A) or a reduced carboxymethylated lactalbumin competition binding assay (26), suggesting that the mutations abrogated intramolecular interaction of the C terminus with the binding pocket. In fact, the apparent affinity of H6DnaK-(387–552)-ye for peptides in the reduced carboxymethylated lactalbumin competition binding assay was similar to that of full-length wild-type DnaK in the high affinity state; peptides V7 (FYQLAKT) and S7K2Y (KYLMFKT) had apparent affinities of 25 and 50 \mu M, respectively, for both full-length DnaK and H6DnaK-(387–552)-ye (26, 27).

Furthermore, unlike H6DnaK-(387–552), in which NR titration at low pH shifted a pre-existing equilibrium between tail-bound and tail-free conformations and resulted in large chemical shift changes for 542 and 543 (25), titration of NR into H6DnaK-(387–552)-ye had no effect on these resonances, indicating that the mutation blocks binding of the C-terminal tail completely (see supplemental data). Importantly, in the context of the two-domain protein DnaK-(1–552)-ye, the same mutations restored a wild-type level of basal ATP hydrolysis, which was stimulated 7-fold by 30 \mu M p5 peptide (Fig. 2), further supporting the interpretation that the binding pocket was empty.

DnaK-(1–552)-ye Was Allosterically Functional in Vitro and in Vivo—Encouraged by the fact that the ATPase rate of DnaK-(1–552)-ye was stimulated by peptide nearly as much as full-length DnaK, we next questioned whether other allosteric properties were preserved. In full-length DnaK, the ATP-induced conformational change to the low affinity state can be monitored via the fluorescence of the single tryptophan (Trp-102) in the ATPase domain. In the presence of ATP, Trp-102 is quenched, blue-shifted, and protected from solvent by a mechanism...
that relies on the presence of a portion of the helical lid subdomain of the SBD (3, 5). Thus, the changes in Trp-102 fluorescence report on ATP-dependent allosteric communication with the SBD. As seen in Fig. 4, the tryptophan fluorescence in DnaK-(1–552)-ye exhibited the same 25% quench and 4-nm blue-shift with ATP binding as in wild-type DnaK. Thus the mutant domain retained all the hallmarks of allostery in DnaK, ATP-induced conformational change to the low affinity state and substrate-induced activation of ATP catalysis. Because these in vitro tests showed DnaK-(1–552)-ye to behave similar to the wild-type protein, we also tested its ability to support replication of bacteriophage λ in vivo. DnaK-(1–552)-ye consistently gave about two-thirds the number of plaques as the wild-type protein, showing that it could substitute for wild-type DnaK in vivo (see supplemental data). Based on these data, which showed the truncated modified SBD to be functional in the context of a two-domain construct, we proceeded to characterize the impact of substrate binding to the isolated SBD using NMR.

NMR Chemical Shift Changes between the Empty and Peptide-bound Forms of H6DnaK-(387–552)-ye Were Primarily Local—TROSY NMR spectra of the empty and NR-bound states of H6DnaK-(387–552)-ye showed similar dispersion and line widths, suggesting that the domain was well folded in both states (Fig. 3A). NR peptide bound stoichiometrically at these concentrations, consistent with the high affinity measured by the reduced carboxymethylated lactalbumin competition binding assay, and it bound in the slow exchange regime, as observed in an earlier NMR study of the isolated β-subdomain (14). However, contrary to that study, in which substantial regions of the empty SBD were broadened beyond detection by conformational exchange, we were able to observe and assign all but three non-proline residues in both states of H6DnaK-(387–552)-ye using 13C/15N-labeled protein and standard methods (see supplemental data).

Based on sequential nuclear Overhauser effects (data not shown), chemical shift indices (see supplemental data), and patterns of hydrogen bonding (Fig. 5), we determined that H6DnaK-(387–552)-ye had the same overall structure as the crystal structure of NR-bound DnaK-(389–607), at least up to residue 537 (10). Therefore, we can discuss chemical shift differences between the NR-bound and empty states in terms of this crystal structure. The chemical shift changes between the NR-bound and empty states were largely local to the crystallographically defined binding pocket (10) and indicated modest remodeling of the hydrophobic binding pocket in the empty state (Fig. 3B). Specifically, most of the large to moderate chemical shift changes were con-
The Empty State of the DnaK Substrate-binding Domain

Peptide binding slows the rate of hydrogen exchange in H6DnaK-(387–552)-ye in the empty (open symbols, circles), 454 (squares), and NR-bound states (filled symbols, squares). Intriguingly, every amide with a measurable exchange rate was less protected in the NR-bound state than in the empty state, consistent with the results of chemical shift analysis. It is interesting to note that, for both NR-bound and empty states, no protection was observed for any residue in the helix region, despite the fact that sequential chemical shift changes relative to the NR-bound state (Δδavg, 0.16 ppm for Ser-423, and the C-terminal residues of the strand) were exchange-broadened beyond detection.

The Empty State Has Faster Hydrogen Exchange Overall—To probe differences in dynamics or overall stability of the domain when empty or bound to substrate, we used hydrogen-deuterium exchange coupled with NMR to monitor rates of amide hydrogen exchange in the empty and NR-bound states. As mentioned previously, the pattern of protected amides for the NR-bound state was consistent with the structure determined crystallographically (Fig. 5) (10). We, furthermore, found that the overall pattern of protected sites in the empty state was similar to the NR-bound state, suggesting that there was no major conformational change in the empty state, consistent with the results of chemical shift analysis. It is interesting to note that, for both NR-bound and empty states, no protection was observed for any residue in the helix region, despite the fact that sequential Δδavg nuclear Overhauser effects (data not shown) and chemical shift indices (see supplemental data) confirm that these residues assume a kinked helical structure, consistent with the conformation observed crystallographically, up to residue Asn-537. However, in the hydrogen exchange experiment, 25 min elapsed between solubilization of the protein in D2O and collection of the first 1-h experiment, so protection of these residues may have been missed. We conclude that the helical region is somewhat metastable. Intriguingly, every amide with a measurable exchange rate was less protected in the empty state than in the NR-bound state, consistent with the idea that peptide binding disfavors open protein conformations that undergo rapid exchange (Fig. 5A). Strand β3 exhibited an alternating pattern of hydrogen exchange protection in the empty state, as expected for the edge strand of a β-sheet (Fig. 5B, top panel) (28). Similar to the chemical shift data, this result is inconsistent with the behavior of this strand in the β-subdomain-only structure, in which the C-terminal half was detached from the rest of the β-sheet in the empty state (14).

The Two Domains of DnaK-(1–552)-ye Do Not Interact in the Absence of Nucleotide—We reasoned that the SBD in isolation might behave differently than when it is linked to or interacting with the ATPase domain. Therefore, we used TROSY NMR to determine whether the...
The Empty State of the DnaK Substrate-binding Domain

conformation of the isolated substrate-binding domain is relevant to its conformation in the context of the ATPase domain. Provocatively, the TROSY spectrum of the DnaK-(1–552)-ye protein overlaid nearly perfectly with those of the isolated ATPase domain and SBD (DnaK-(1–388) and H6DnaK-(387–552)-ye, respectively; Fig. 6), indicating that there was very little interaction between the two domains. The same was true even when the SBD binding pocket was saturated with NR peptide; the spectrum of the NR-bound two-domain construct overlaid with those of the isolated ATPase domain and the isolated NR-bound SBD (data not shown). Apparently, nucleotide must be present in order for stable domain docking to occur. Taken altogether, these data argued strongly that DnaK-(1–552)-ye was a functional protein and that residues 387–552 assumed the same conformation in isolation as they did when the ATPase domain was present.

DISCUSSION

In this work, we have engineered a truncated DnaK SBD construct, H6DnaK-(387–552)-ye, which could be observed in both the empty and peptide-bound conformations. This domain existed in a high affinity conformation in the absence of influence from the ATPase domain, consistent with previous studies on the full-length SBD (3,29). Thus, the presence of the ATPase domain is absolutely required to convert the SBD to its low affinity conformation. Hydrogen exchange measurements and NMR chemical shift dispersion demonstrated that H6DnaK-(387–552)-ye formed a well folded, stable structure in the presence and absence of bound peptide. NMR data for the peptide-bound state were entirely consistent with the x-ray crystal structure of the full SBD bound to peptide (10). The conformational change from the peptide-bound state to the empty state was local in scope, primarily involving residues that directly flank the peptide-binding pocket, although there also appeared to be a change in packing of the helix against the β-subdomain. This characterization of the empty state of H6DnaK-(387–552)-ye is at odds with an earlier study of an isolated β-subdomain, which was truncated at residue 507 (Fig. 1A) and therefore lacked all of the α-helix (14). In this smaller construct, many residues in the empty state were broadened beyond detection by conformational exchange, the C-terminal half of strand β3 detached from its sheet to move into the peptide-binding pocket, and the affinity for peptide was extremely low (Kd of 0.6 mM for NR). We conclude that the structure of the isolated β-subdomain was compromised either by removal of the entire α-helical subdomain or the fact that it existed as a dimer in the empty state (17).

When we looked at our modified SBD in a two-domain construct that included the ATPase domain (i.e. DnaK-(1–552)-ye), the typical indicators of DnaK allostery were preserved, a low basal ATPase rate that was stimulated by peptide and an ATP-induced blue shift of Trp-102 that reported on the conversion of the SBD to the low affinity state. This was not an unexpected result, as many studies have reported wild-type function of slightly shorter fragments of DnaK and BiP that end near residue 538 (DnaK numbering) (3, 5, 6, 12, 30).

One of the most striking conclusions from this work, however, stems from the fact that the TROSY NMR spectrum of DnaK-(1–552)-ye overlaid perfectly with the spectra of the ATPase and SBD domains in isolation, arguing that the two domains acted independently when they were linked, whether or not peptide was bound. We conclude that nucleotide must be bound in order for the two domains to dock. One might question the physiological significance of the no-nucleotide state of DnaK, but in fact, our more recent results have demonstrated that, even in the presence of ADP, the two domains do not interact; only ATP can induce a docked conformation. This behavior is consistent with a host of biochemical evidence that shows the ADP-bound and no-nucleotide states of DnaK to be very similar, with only ATP causing interdomain effects (3, 5, 31, 32). However, this result is in contrast to a recent NMR report of domain docking in both ADP- and ATP-bound states of a two-domain construct of Thermus thermophilus DnaK that lacked the entire α-helical lid of the SBD (corresponding to 1–507 in E. coli DnaK numbering) (33). The reasons for this difference are not yet clear, but we speculate that the T. thermophilus construct was predisposed to a low affinity conformation because of mutations/deletions in the peptide binding pocket and/or removal of the entire α-helical lid subdomain. Based on the biochemical measures of two-way allostery, which showed peptide binding influencing the activity of the ATPase domain, we expected peptide binding to the SBD to cause a ripple of conformational changes propagating into the ATPase domain. On the contrary, however, peptide binding to the nucleotide-free two-domain construct only caused very local intradomain effects, and there was no evidence for chemical shift changes at residue Lys-414 or in the linker VLLL sequence, both of which have been shown by mutagenesis to be essential to this allosteric mechanism (Fig. 1A) (18, 34). Importantly, every assay we are aware of that shows peptide binding causing an effect in the ATPase domain is (necessarily) conducted in the presence of ATP (35–37). Therefore, we hypothesize that an interface with the ATP-bound ATPase domain is also required for propagation of an allosteric signal from peptide binding to the ATPase domain. We intend to pursue these intriguing observations by identifying functional docking interfaces through evolutionary conservatism of interdomain thermodynamic couplings and continued study by NMR.

FIGURE 6. The two domains of DnaK-(1–552)-ye behave independently. TROSY NMR spectrum of 0.25 mM 15N-DnaK-(1–552)-ye (red outlines) overlaid with TROSY spectra of 0.7 mM 15N-H6DnaK-(387–552)-ye in blue and 0.35 mM 15N-DnaK-(1–388) in green, 30 °C.

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The Empty State of the DnaK Substrate-binding Domain

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