A disulfide-bonded DnaK dimer is maintained in an ATP-bound state

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Abstract DnaK, a major Hsp70 molecular chaperones in *Escherichia coli*, is a widely used model for studying Hsp70s. We recently solved a crystal structure of DnaK in complex with ATP and showed that DnaK was packed as a dimer in the crystal structure. Our previous biochemical studies supported the formation of a specific DnaK dimer as observed in the crystal structure in solution in the presence of ATP and suggested an important role of this dimer in efficient interaction with Hsp40 co-chaperones. In this study, we dissected the biochemical properties of this DnaK dimer. To restrict DnaK in this dimer form, we mutated two residues on the dimer interface to cysteine, A303C, and H541C. Upon oxidation, this DnaK-A303C-H541C protein formed a specific dimer linked by disulfide bonds formed between A303C and H541C only in the presence of ATP, consistent with the crystal structure. Intriguingly, this disulfide-bond-linked dimer of DnaK-A303C-H541C has reduced ATPase activity and decreased affinity for peptide substrate. More interestingly, unlike wild-type DnaK, the peptide substrate-binding kinetics of this dimer is drastically accelerated even in the

absence of ATP, suggesting this dimer is restricted in an ATP-bound conformation regardless of nucleotide bound, which was further supported by our analysis using tryptophan fluorescence and ATP-induced peptide release. Thus, formation of the dimer restricted DnaK in an ATP-bound state and blocked the progression through the chaperone cycle. Productive progression through the chaperone cycle requires the dissociation of this transient dimer. Surprisingly, a significantly compromised interaction with Hsp40 co-chaperone was observed for this disulfide-bond-linked dimer. Thus, dissociation of this DnaK dimer is equally crucial for efficient Hsp40 interaction. An initial interaction between Hsp70 and Hsp40 requires the formation of DnaK dimer; but a stable Hsp70-Hsp40 interaction may follow the dissociation of the dimer.

Keywords HSP70 · Hsp40 · Molecular chaperone · ATPase · Peptide substrate binding · Protein folding

Introduction

Heat shock proteins 70 kDa (Hsp70s) are ubiquitous molecular chaperones across all kingdoms of life (Boorstein et al. 1994; Bukau et al. 2000; Mayer and Bukau 2005; Bukau et al. 2006; Eisenberg and Greene 2007; Hartl and Hayer-Hartl 2009; Young 2010). They play essential roles in every aspect of cellular protein homeostasis including protein folding, transportation across membrane, assembly, degradation, and quality control. Due to the fundamental importance of maintaining protein homeostasis, Hsp70s are connected with many human diseases including various cancers and neurodegenerative diseases (Muchowski and Wacker 2005; Brodsky and Chiosis 2006; Balch et al. 2008; Evans et al. 2010; Murphy 2013).

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Hsp70s are highly conserved. Even between the most distant species such as *Escherichia coli* and humans, there is about 40% sequence identity (Boorstein et al. 1994). Every Hsp70 molecule is composed of two functional domains: nucleotide-binding domain (NBD) at the N-terminus and substrate-binding domain (SBD) at the C-terminus (Bukau and Horwich 1998; Mayer and Bukau 2005; Hartl and Hayer-Hartl 2009; Young 2010). These two functional domains are connected by a short linker segment called inter-domain linker. NBD binds ATP and hydrolyzes it to ADP, providing energy to power the chaperone activity (Flynn et al. 1989). SBD recognizes and binds hydrophobic segments of polypeptides in extended conformations (Landry et al. 1992; Blond-Elguindi et al. 1993; Gragerov et al. 1994; Zhu et al. 1996; Rudiger et al. 1997). Although each domain can bind their substrates independently, chaperone activity requires a tight coupling between them. ATP binding to NBD drastically accelerates the on and off rates of polypeptide substrate binding to SBD with the off rate being increased more (Flynn et al. 1989; Schmid et al. 1994). Thus, the affinity of polypeptide substrate in the ATP-bound state is lower than that of the ADP-bound state, by about 2–3 orders of magnitude. At the same time, polypeptide substrate binding stimulates the ATP hydrolysis rate of NBD which normally is quite low (Flynn et al. 1989). This ATP-induced allosteric coupling makes sure the energy from ATP hydrolysis is efficiently used to regulate polypeptide substrate binding for the powerful chaperone activity.

Structures of the isolated domains have been known for decades. These structures have shown the structural basis of each domain in binding its substrates. The NBD is made of two lobes, between which is the nucleotide-binding pocket (Flaherty et al. 1990; Harrison et al. 1997; Sriram et al. 1997; Jiang et al. 2007; Wisniewska et al. 2010). SBD is further divided into two subdomains: SBD β and SBD α (Zhu et al. 1996; Cupp-Vickery et al. 2004; Zhang et al. 2014; Clerico et al. 2015). A single peptide-binding site is formed between two loops in SBD β with SBD α covering the peptide-binding site as a lid. Three recent crystal structures of Hsp70s containing both functional domains were solved in complex with ATP including two structures of DnaK, an *E. coli* Hsp70, and a structure of human BiP, an ER Hsp70 (Kityk et al. 2012; Qi et al. 2013; Yang et al. 2015). Together with a number of recent NMR studies (Zhuravleva et al. 2012; Zhuravleva and Gierasch 2015), these structures have provided breakthrough insights into the essential ATP-induced allosteric coupling. Interestingly, in all these three structures, Hsp70s are packed as a dimer. Historically, Hsp70s have been shown to behave mainly as monomer in the presence of ATP and form low-ordered oligomer in the absence of ATP (Carlino et al. 1992; Osipiuk et al. 1993; Benaroudj et al. 1995; Schonfeld et al. 1995; Gao et al. 1996; Liu et al. 2003; Preissler et al. 2015).

Our biochemical analysis inspired by the structure of DnaK in complex with ATP, DnaK-ATP, suggested that DnaK forms specific dimer in the presence of ATP in solution although the amount is low, and this dimer is crucial for both chaperone activity and the efficient interaction with Hsp40 co-chaperones (Sarheng et al. 2015). Consistent with this study, a report from Gestwicki's group also demonstrated that DnaK forms a small amount of dimer in the presence of ATP (Thompson et al. 2012). However, the role of this DnaK-ATP dimer in the chaperone cycle remains elusive.

In general, Hsp70 chaperone activity requires two types of cofactors: Hsp40 chaperones and nucleotide-exchange factors (NEFs) (Liberek et al. 1991; Schroder et al. 1993; Fan et al. 2003; Mayer and Bukau 2005; Hendrickson and Liu 2008; Hartl and Hayer-Hartl 2009; Kampinga and Craig 2010; Young 2010). Hsp40s have been shown to stimulate the ATP hydrolysis step of Hsp70s while NEFs speed up the release of ADP and binding of ATP after ATP hydrolysis in Hsp70s. Although essential and functionally conserved, Hsp40s are more diverse than Hsp70s. Some Hsp40s can bind both unfolded and partially folded polypeptides directly and have been proposed to bring polypeptide substrates to Hsp70s upon interacting with Hsp70s. All Hsp40s contain a conserved domain called J-domain, named after DnaJ, a well-studied Hsp40 from *E. coli*. Crucial for stimulating the ATPase activity of Hsp70s, the J-domain has been suggested to directly interact with the bottom cleft of the NBD of Hsp70s (Gassler et al. 1998; Greene et al. 1998; Suh et al. 1998; Davis et al. 1999; Wisen et al. 2010; Kim et al. 2014). However, due to the transient nature of Hsp70-Hsp40 interaction, the molecular mechanism of this interaction is largely unknown (Alderson et al. 2016).

In this study, we dissected the biochemical properties of the DnaK-ATP dimer in order to characterize its role in the Hsp70 chaperone cycle and Hsp70-Hsp40 interaction. First, we mutated two residues on the dimer interface in DnaK to cysteine: Ala303 and His541. Upon oxidation, A303C forms a specific disulfide bond with H541C from another DnaK molecule only in the presence of ATP. Thus, a DnaK dimer linked through two A303C-H541C disulfide bonds is formed specifically in the presence of ATP. Interestingly, this DnaK-A303C-H541C dimer has a reduced ATP hydrolysis rate and affinity for peptide substrates. More importantly, this dimer appears to be restricted in an ATP-bound conformation regardless of nucleotides bound to NBD. Restricting DnaK to this dimer drastically compromises Hsp40 interaction and chaperone activity. Taken together, (1) ATP binding induces the formation of DnaK-ATP dimer, and this dimer slows down ATP hydrolysis to prevent non-productive ATP hydrolysis, and (2) stable interaction with Hsp40 and efficient progress through chaperone cycle requires the dissociation of this dimer.

Methods

Protein expression and purification

The DnaK proteins used in this work, WT, C15A, and C15A-A303C-H541C, were over-expressed from a *dnak* expression plasmid pBB46 (*amp^R*) in a *dnak* deletion strain BB205 (*cam^R* *kan^R*) (Burkholder et al. 1996). The mutations were introduced into DnaK using QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies). All the expressed DnaK proteins have a 6-Histidine tag was added at the C-terminus to facilitate protein purification. Expression was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) in LB medium for 5 h at 30 °C. The purification was carried out as described previously (Kumar et al. 2011; Qi et al. 2013; Sarbeng et al. 2015). The purified proteins were concentrated to >10 mg/ml in buffer containing 10 mM Hepes-KOH, pH 7.5, 50 mM KCl, and 1 mM DTT, flash frozen in liquid nitrogen and stored in -80 °C freezer.

The DnaJ and GrpE proteins used in the refolding assay were purified as described before using the pSMT3 vector (Mossessova and Lima 2000; Kumar et al. 2011; Sarbeng et al. 2015).

Disulfide crosslinking with Cu phenanthroline

The oxidation for forming disulfide bond was performed as described before (Sarbeng et al. 2015). Briefly, purified DnaK-C15A-A303C-H541C protein was diluted to 5 mg/ml in buffer A (25 mM Hepes-KOH, pH 7.5, 150 mM KCl, 10 mM Mg(OAc)₂, and 10% glycerol) with addition of 5 mM DTT. After reducing all the cysteine residues by incubating on ice for 2 h, DTT was removed by a spin column pre-equilibrated with buffer A. Protein was diluted to 0.5 mg/ml with buffer A and freshly mixed 100 μ M CuSO₄, and 200 μ M 1,10-phenanthroline was added and incubated for 1 h on ice. EDTA was added to a final concentration of 5 mM to terminate the oxidation. Oxidation was analyzed with SDS-PAGE without reducing agent.

Purification of the specific dimer of DnaK-C15A-A303C-H541C formed in the presence of ATP

To purify the specific dimer of DnaK-C15A-A303C-H541C protein formed in the presence of ATP, the DnaK-C15A-A303C-H541C protein was first purified as described above under reducing condition. Then, the purified DnaK-C15A-A303C-H541C protein was incubated with 5 mM DTT in buffer A on ice for 2 h to fully reduce the cysteine residues. After removing DTT using desalting column, the DnaK protein was diluted to 0.5 mg/ml using buffer A with 2 mM ATP

and oxidized with 50 μ M copper phenanthroline on ice for 1 h to form the specific dimer. The efficiency of dimer formation was checked by running SDS-PAGE in the absence of reducing reagent. After precipitating with 80% ammonium sulfate, the oxidized dimer protein was purified on a hydrophobic column HiTrap Butyl HP with ammonium sulfate gradient. Fractions corresponding to dimer on nonreducing SDS-PAGE were pooled, and ATP was removed by dialysis. Before storing in -80 °C freezer, the purified dimer was concentrated to >10 mg/ml and flash frozen in liquid nitrogen. We label this dimer as 303/541 (dimer) in all the figures.

Single-turnover ATPase assay for rate of ATP hydrolysis (k_{cat})

Single-turnover ATPase assay on all the DnaK proteins was performed practically as described previously with modification (Liu et al. 2001; Kumar et al. 2011). Briefly, DnaK proteins were incubated with [α -³²P] ATP (NEG503H250UC, 3000 Ci/mmol; Perkin Elmer) in buffer A to form DnaK-ATP complex. Then, a spin column was used to quickly isolate the DnaK-ATP complexes from free ATP. The complexes were frozen in liquid nitrogen. The single-turnover reactions were started by mixing equal volume of DnaK-ATP complex with equal volume of buffer. After incubating at 20° for indicated time periods, samples were taken and PEI-cellulose thin-layer chromatography plates (Sigma-Aldrich) was used to separate ATP from ADP. A Typhoon phosphorimaging system (GE Healthcare) was used to visualize and quantify the amount of radioactive ATP and ADP, and GraphPad Prism was used to determine k_{cat} after data fitting using first-order rate equation.

Fluorescence anisotropy assay for peptide substrate binding

Model peptide NR (sequence: NRLLLTG) was purchased from NEOBioscience with greater than 95% purity. To facilitate fluorescence anisotropy assay, the NR peptide was labeled with fluorescein at the N-terminus. Serial dilutions of DnaK proteins were incubated with 20 nM NR in buffer A with addition of 100 μ M ADP for more than 3 h to allow binding to reach equilibrium. All the fluorescence anisotropy measurements were taken on a Beacon 2000 instrument (Invitrogen), and data were fitted to a one-site binding equation using PRISM (GraphPad) to deduce dissociation constants (K_d).

To determine the binding kinetics, DnaK proteins (final concentration, 15 μ M) was mixed with F-NR peptide at 20 nM final concentration in buffer A. Right after mixing, fluorescence anisotropy measurements were taken every 10 s to track the binding kinetics. For the sample with ATP, 2 mM ATP was included in the buffer.

For measuring ATP-induced release of bound NR peptide, DnaK-NR complex was first formed by incubating 15 μ M DnaK with 20 nM NR in buffer A containing 100 μ M ADP for more than 3 h to allow binding to reach equilibrium. Fluorescence anisotropy was measured every 10 s to show the binding has reached equilibrium. Then, right after ATP was added to a final concentration of 2 mM, fluorescence anisotropy measurements were taken every 10 s to track the release of bound NR peptide.

Tryptophan fluorescence assay for ATP-induced allosteric coupling

The emission spectra of tryptophan fluorescence were measured as described previously (Kumar et al. 2011; Qi et al. 2013). Basically, all the purified DnaK proteins were diluted to 1 μ M using buffer A. ATP or ADP was added to a final concentration of 2 mM. After incubating for 2 min, emission spectra from 310 to 400 nm with excitation at 295 nm were collected. The peaks were determined.

Surface plasmon resonance analysis of the DnaK-DnaJ interaction

A Biacore T200 system (GE Healthcare) was used to analyze the DnaK-DnaJ interaction, and the surface plasmon resonance (SPR) assay was carried out as described in previous studies (Kumar et al. 2011; Sarbeng et al. 2015). The DnaJ-BCCP construct was a generous gift from Dr. Elizabeth Craig. After overexpression in BL21(Gold), cell lysate expressing DnaJ-BCCP fusion protein was prepared by sonication (Suh et al. 1998; Davis et al. 1999). About 200 response units of this DnaJ-BCCP protein was immobilized on a SA sensor chip. All the SPR assay were performed with 2 μ M DnaK protein using buffer A including 0.003% P20 and 1 mM ATP. The first channel was coated with biotin and used as a background control. All plotted traces were retrieved after subtracting this background control.

Luciferase refolding assay for in vitro chaperone activity

The refolding assay with heat-denatured luciferase for the DnaK-DnaJ-GrpE chaperone machinery was carried out as described in previous studies (Qi et al. 2013; Sarbeng et al. 2015). Briefly, purified Firefly luciferase (Promega) was denatured by incubating at 42 °C for 20 min and diluted into a refolding reaction buffer containing 3 μ M DnaK, 0.66 μ M DnaJ, and 0.33 μ M GrpE in buffer B (25 mM Hepes-KOH, pH 7.5, 100 mM KOAc, 10 mM Mg(OAc)₂, and 3 mM ATP). The luciferase activity was measured by a luminometer (Berthold LB9507) at indicated time points. The unheated luciferase was used as positive control, and the activity was set as 100%.

Growth test

The growth test was performed as described previously (Kumar et al. 2011; Qi et al. 2013; Sarbeng et al. 2015). The mutations were introduced into a *dnak* expression plasmid pBB46 using the Quik-change Lightening Mutagenesis kit (Stratagene) and transformed in a *dnak* deletion strain BB205 (cam^R kan^R) (Burkholder et al. 1996). Growth test was done with fresh overnight culture on LB plate containing 50 μ g/ml Amp, 25 μ g/ml Kan, and 25 μ g/ml Cam and 20 μ M IPTG.

Results

DnaK forms a specific dimer linked by two disulfide bonds formed between A303C and H541C in the presence of ATP

DnaK is a well-studied model for Hsp70s. Recently, we have shown that DnaK forms a dimer in the DnaK-ATP crystal structure and this dimer forms in solution although the amount is low (Sarbeng et al. 2015). Ala303 and His541 are on the dimer interface (Fig. 1). In our previous studies, we have generated two mutant DnaK proteins: DnaK-A303C and DnaK-H541C by mutating Ala303 and His541 individually to cysteine. Upon oxidation, these two mutant proteins formed a specific dimer only in the presence of ATP. To restrict DnaK to a dimer seen in the DnaK-ATP structure, we introduced both A303C and H541C to the same DnaK construct and purified the double cysteine mutant: DnaK-A303C-H541C. DnaK has a single endogenous cysteine, Cys15. To prevent Cys15 from forming nonspecific disulfide bonds with either A303C or H541C, it was mutated to alanine, which has been shown to have a limited effect in either the biochemical or chaperone activity of DnaK (Mapa et al. 2010). We have purified both wild-type (WT) DnaK and DnaK-C15A proteins and used them as controls. All the DnaK proteins were highly purified with minimal contamination (Fig. 2a).

Consistent with our hypothesis, upon oxidation by copper phenanthroline, the DnaK-C15A-A303C-H541C mutant protein formed a specific dimer band only in the presence of ATP (Fig. 2b). In the presence of ADP, many bands were formed, suggesting formation of many nonspecific disulfide bonds. We first oxidized DnaK-C15A-A303C-H541C protein in the presence of ATP to form the specific dimer, and then purified this specific dimer to high purity (303/541(dimer) in Fig. 2a). Upon reduction with DTT, this dimer was dissociated back to monomer, consistent with the formation of specific disulfide bonds. Previously, we have reported that purified DnaK-C15A-A303C and DnaK-C15A-H541C proteins formed a specific dimer through a disulfide bond between A303C and H541C upon oxidation only in the presence of ATP (Sarbeng

Fig. 1 Ala 303 and His541 are on the dimer interfaces of the DnaK-ATP structure. Ribbon diagram of the DnaK-ATP dimer structure. The two protomers are labeled as A and B. The domain coloring is NBD (blue for A and light blue for B), inter-domain linker (purple for A and light purple for B), SBD β (green for A and light green for B), and SBD α (red for A and pink for B). Ala303 and His 541 are highlighted in space-fill presentation

et al. 2015). The DnaK-C15A-A303C-H541C dimer showed a similar molecular mass as the dimer formed between DnaK-C15A-A303C and DnaK-C15A-H541C proteins, suggesting the formation of disulfide bonds between A303C and H541C in the DnaK-C15A-A303C-H541C dimer. Moreover, peptide mass fingerprinting analysis after trypsin digestion supported

the formation of specific disulfide bonds between A303C and H541C. Thus, we have successfully formed a DnaK dimer using DnaK-C15A-A303C-H541C, most likely as seen in the DnaK-ATP structure (Fig. 1).

The disulfide-bonded DnaK dimer has reduced intrinsic ATPase activity and affinity for peptide substrate

Now, we have shown that to be able to restrict DnaK in a specific dimer linked by disulfide bonds using DnaK-C15A-A303C-H541C, we first formed a large amount of this dimer in the presence of ATP, and then purified this dimer. Next, we characterized the biochemical properties of this dimer. We first looked at the intrinsic ATPase rate using a single-turnover ATPase assay (Davis et al. 1999; Kumar et al. 2011). WT DnaK and DnaK-C15A were used as controls (Fig. 3a, c). Consistent with a previous report (Mapa et al. 2010), C15A only had a minor influence on the ATPase rate. Under reducing condition, the ATPase rate of DnaK-C15A-A303C-H541C was similar to that of DnaK-C15A, suggesting neither A303C nor H541C mutation has appreciable effect on the intrinsic ATPase activity. Interestingly, the ATP hydrolysis rate of the disulfide-bonded dimer of DnaK-C15A-A303C-H541C was reduced by more than 3-fold compared with the reduced form (Fig. 3a, c). Thus, forming the dimer resulted in a reduction in intrinsic ATPase activity.

Fig. 2 The DnaK-C15A-A303C-H541C protein forms specific dimer only in the presence of ATP. **a** Purified DnaK proteins. WT wild-type DnaK. C15A DnaK-C15A, 303/541 (red) DnaK-C15A-A303C-H541C purified under reducing condition, 303/541 (dimer) + DTT purified DnaK-C15A-A303C-H541C protein was oxidized to form dimer in the presence of ATP; after the dimer was purified, DTT was added to reduce the disulfide bonds back to monomer to show that the dimer was formed

from disulfide bonds, 303/541 (dimer) after purified, DnaK-C15A-A303C-H541C was oxidized to dimer in the presence of ATP, the dimer was purified and loaded on to SDS-PAGE. **b** Oxidation of DnaK-C15A-A303C-H541C protein. Purified DnaK-C15A-A303C-H541C was oxidized with copper-phenanthroline in the presence of ATP or ADP. The position of the specific dimer formed in the presence of ATP was indicated by an arrow

Fig. 3 Both the intrinsic ATPase activity and peptide substrate-binding affinity were compromised for the dimer of DnaK-C15A-A303C-H541C. **a** The intrinsic ATPase activity. A single-turnover ATPase assay was used to determine the intrinsic ATPase activity of DnaK proteins. **b** Fluorescence polarization assay for determination of peptide substrate-binding affinity in the presence of ADP. Model peptide NR was labeled with fluorescein (F-NR) and incubated with serial dilutions of DnaK

proteins in the presence of 100 μM ADP. **c** Summary of the ATPase rate (k_{cat}) and affinity of peptide NR binding (K_d). k_{cat} and K_d values were calculated from (a) and (b), respectively. WT wild-type DnaK, C15A DnaK-C15A, 303/541(red) DnaK-C15A-A303C-H541C purified under reducing condition, 303/541(dimer) purified specific dimer of DnaK-C15A-A303C-H541C formed in the presence of ATP

Next, we analyzed the intrinsic peptide substrate-binding activity using fluorescence polarization assay. In this assay, a model peptide NR (Gragerov et al. 1994; Zhu et al. 1996), a well-established peptide substrate for DnaK, was labeled at N-terminus with fluorescein. The intrinsic peptide substrate-binding activity is represented by the peptide substrate-binding affinity in the presence of ADP. Consistent with published results (Kumar et al. 2011; Xu et al. 2012), the binding affinity for this F-NR peptide for WT DnaK was around 1.69 μM in the presence of ADP (Fig. 3b, c). Under reducing condition, neither C15A nor the C15A-A303C-H541C influenced the affinity for F-NR peptide significantly, suggesting none of the mutations had significant effect on the affinity for peptide substrate. It was expected for C15A and A303C since both Cys15 and Ala303 are in NBD. Although His541 is on SBD α , it is too far away from the substrate-binding site to cause any appreciable effect. Intriguingly, the affinity for NR peptide was reduced by more than 8-fold for the disulfide-bonded dimer of DnaK-C15A-A303C-H541C in the presence of ADP, suggesting that restricting DnaK into dimer reduces the affinity for peptide substrate.

The reduced affinity for peptide NR could be caused by altered kinetics of peptide substrate binding. Then, we tested peptide-binding kinetics. For the WT DnaK, the peptide NR binding showed slow kinetic in the presence ADP but much faster in the presence of ATP (Fig. 4a, b) as reported previously (Flynn et al. 1989; Schmid et al. 1994). Consistent with the near WT-level affinity for NR peptide, both C15A and reduced C15A-A303C-H541C behaved like WT DnaK: slow

kinetic in the presence of ADP and much faster kinetics in the presence of ATP, suggesting these mutations by themselves have little influence on the kinetics of peptide substrate binding. In contrast, the oxidized dimer of C15A-A303C-H541C showed remarkably fast binding kinetics even in the presence of ADP as well (Fig. 4a, b), suggesting the dimer is locked in an ATP-bound conformation even in the absence of ATP.

Fig. 4 Kinetics of peptide NR binding to DnaK proteins in the presence of ADP (a) and ATP (b), respectively. DnaK proteins (15 μM) was first incubated with ADP or ATP for 2 min. Then, F-NR was added and the binding was recorded over time. The names of DnaK proteins are the same as in Fig. 3

The dimer of DnaK-C15A-A303C-H541C is maintained in an ATP-bound state regardless of nucleotide bound

To confirm the DnaK-C15A-A303C-H541C dimer is restrained in an ATP-bound state regardless of nucleotide being bound to NBD, we analyzed the ATP-induced allosteric coupling using two approaches: tryptophan fluorescence and bound-peptide release induced by ATP binding.

DnaK has a single tryptophan, Trp102, in the NBD. The fluorescence of Trp102 is sensitive to the nucleotide-binding state: in the presence of ATP, the peak is shifted to shorter wavelength than that in the presence of ADP or in the absence of nucleotide (Palleros et al. 1993; Buchberger et al. 1995). This blue shift of tryptophan fluorescence spectrum is caused by the docking of SBD α to NBD around the site of Trp102 upon ATP binding to DnaK (Kityk et al. 2012; Qi et al. 2013). Thus, in the presence of ATP, Trp102 is more buried relative to that of in the presence of ADP or no-nucleotide-added conditions. This blue shift has been used as an indication of ATP-induced allosteric coupling in DnaK. Consistent with previous observations, the peak of WT DnaKs tryptophan fluorescence was at ~342 and ~335 nm in the presence of ADP and ATP, respectively (Fig. 5a). ADP condition was very similar to that of the no-nucleotide-added condition. Thus, there was a blue shift. C15A mutation had no observable influence in this ATP-induced blue shift. Under the reducing condition, the DnaK-C15A-A303C-H541C protein also behaved similarly as WT DnaK, suggesting a close to WT-level ATP-induced allosteric coupling. Thus, none of the mutations by itself influenced the ATP-induced allosteric coupling appreciably. In contrast, the disulfide-bonded dimer of DnaK-C15A-A303C-H541C protein demonstrated a peak around 334 to 335 nm no matter what nucleotide was present, supporting our hypothesis that the dimer is maintained in an ATP-bound state regardless of the nucleotide-bound state of NBD.

Next, we tested the ATP-induced release of bound peptide using NR peptide as a substrate. We first formed the complexes between DnaK proteins and F-NR peptide in the presence of low concentration of ADP. After the binding reached equilibrium, 2 mM ATP was added to each complex, and the release of bound NR peptide was recorded over time. Before addition of ATP, all the DnaK proteins bound F-NR peptide stably (Fig. 5b). Consistent with previous reports (Montgomery et al. 1999; Pellicchia et al. 2000; Kumar et al. 2011), bound F-NR peptide was quickly released from WT DnaK. Neither C15A nor the reduced C15A-A303C-H541C protein showed significant difference from WT DnaK, consistent with the observation from the above tryptophan fluorescence assay. Interestingly, little release of bound NF peptide from the dimer of C15A-A303C-H541C was observed right after addition of ATP. Over time, a very slow release was detected. Taken together, the dimer of DnaK-C15A-A303C-H541C is restrained in an ATP-bound state, most likely as in the DnaK-ATP structure.

Fig. 5 The ATP-induced allosteric coupling in DnaK proteins. **a** The tryptophan fluorescence of DnaK proteins. Tryptophan fluorescence spectra of DnaK proteins (1 μ M) were collected with excitation at 295 nm in absence of nucleotide (Apo) and in the presence of ATP and ADP. The peak value for each spectrum is the wavelength of the maximal emission. **b** The ATP-induced release of bound NR peptide from DnaK proteins. F-NR peptide was incubated with DnaK proteins (15 μ M) for more than 3 h to allow binding to reach equilibrium. Then, ATP was added (indicated by an arrow at time zero), and the release of bound NR peptide was recorded over time. The names of DnaK proteins are the same as in Fig. 3

The interaction with Hsp40 co-chaperone DnaJ was significantly compromised by the dimer formation

Previously, we have shown that the DnaK dimer observed in the DnaK-ATP structure is essential for efficient interaction with Hsp40 co-chaperone DnaJ since mutations compromised the formation of DnaK dimer, showing a reduced Hsp40 interaction (Sarheng et al. 2015). Now, we have used disulfide bonds to restrain DnaK in such a dimer. Next, we tested how this dimer interacts with DnaJ. It is possible that this dimer has an enhanced interaction with DnaJ since compromising the dimer reduced DnaK-DnaJ interaction. On the other hand, the dimer showed a reduced ATPase rate and binding affinity for peptide substrates. Both activities seem to be crucial for the efficient DnaK-DnaJ interaction (Wawrzynow and Zylicz 1995; Gassler et al. 1998; Suh et al. 1998, 1999; Davis et al. 1999). Moreover, previous mutational studies have shown that ATP binding is not sufficient for a stable DnaK-DnaJ interaction (Suh et al. 1999). Maybe simply maintaining an ATP-bound conformation is not sufficient for a stable DnaK-DnaJ interaction.

To analyze the DnaK-DnaJ interaction, we took advantage of the surface plasmon resonance assay. A DnaJ construct

fused to BCCP was immobilized on a sensor chip. Consistent with previous observations (Wawrzynow and Zylicz 1995; Suh et al. 1998, 1999; Laufen et al. 1999, Mayer et al. 1999), a robust interaction was observed when WT DnaK was flowing through the sensor chip in the presence of ATP (Fig. 6a). DnaK-C15A had a resonance signal about 70% of that WT DnaK regardless of the presence of reducing reagents. A303C-H541C under reducing condition further decreased the resonance signal close to 70% of the C15A control. This is reasonable since both Ala303 and His541 are on the dimer interface. Mutating both of them to cysteine may compromise the formation of dimer to some extent. Intriguingly, the disulfide-bonded dimer of DnaK-C15A-A303C-H541C drastically reduced the resonance signal to less than 10% of those of the C15A control and WT DnaK. Thus, restraining DnaK into a dimer as observed in the DnaK-ATP structure significantly compromise the DnaK-DnaJ interaction.

Restricting DnaK in dimer abolishes chaperone activity

Now, we have characterized the biochemical properties of the dimer of DnaK-C15A-A303C-H541C. Based on the biochemical properties, this dimer is most likely maintained in an ATP-bound conformation as observed in our DnaK-ATP structure, and its interaction with DnaJ is drastically compromised. Since the chaperone activity of DnaK depends on a normal chaperone cycle between ATP- and ADP-bound states and also DnaJ interaction, we expected that the chaperone

activity of the dimer would be significantly compromised. To test this hypothesis, we carried out luciferase refolding assay to analyze the chaperone activity of DnaK in refolding heat-denatured luciferase. As expected, WT DnaK refolded back almost all of the denatured luciferase within 30-min incubation with the DnaK chaperone machinery including DnaK and its two co-chaperones DnaJ and GrpE, the NEF for DnaK (Fig. 7a). C15A had little influence on the refolding activity of DnaK. Although the ATPase activity of DnaK-C15A was slightly slower than that of WT and the DnaJ interaction was about 70% of that of the WT DnaK, these defects most likely were not sufficient to cause an observable chaperone activity defect in refolding luciferase. However, the growth of *E.coli* harboring this mutant DnaK was compromised, indicating a moderate defect in in vivo chaperone activity (Fig. 7b). The reduced DnaK-C15A-A303C-H541C protein showed a significant decrease in refolding heat-denatured luciferase, only about 50% of the WT activity. Consistent with this defect, the growth of *E.coli*, whose cytoplasm is reducing, carrying this mutant was significantly compromised although it grew better than the vector control, the negative control. This chaperone activity defect is consistent with the DnaJ interaction defect observed above, which is a combination of all the three mutations including the C15A in DnaK. Importantly, forming disulfide-bonded dimer in DnaK-C15A-A303C-H541C completely abolished the refolding activity, supporting our hypothesis that restraining DnaK in an ATP-bound state dimer is detrimental to chaperone activity. Since the cytoplasm of *E.coli* is reducing, we were not able to test the in vivo effect of the disulfide-bonded dimer of DnaK-C15A-A303C-H541C.

Fig. 6 The dimer of DnaK-C15A-A303C-H541C has a drastically reduced interaction with DnaJ. The DnaK-DnaJ interaction was assayed using surface plasmon Resonance under reducing condition (in the presence of DTT (a)) and oxidizing condition (in absence of DTT (b)); ~200 response units of DnaJ was immobilized on a sensor chip. Then, DnaK proteins at 2 μ M were run through the sensor chip. Resonance signals were recorded over time after subtracting the background signals from a control channel. The names of DnaK proteins are the same as in Fig. 3

Discussion

In this study, we have used disulfide-bonds between A303C and H541C to restrict DnaK into a dimer based on our recently published DnaK-ATP structure, and characterized the biochemical properties of this dimer. Interestingly, our biochemical data including peptide-binding kinetics, tryptophan fluorescence and bound-peptide release induced by ATP all supported that the dimer is locked in an ATP-bound state most likely as observed in the DnaK-ATP structure regardless of nucleotide bound to the NBD. The results of tryptophan fluorescence can be readily interpreted. The tryptophan fluorescence spectrum measures the NBD-SBD α contacts since the only tryptophan, Trp102 (in NBD), is buried on the NBD-SBD α interface in the ATP-bound state, but is free in the ADP-bound and nucleotide-free states (Buchberger et al. 1995; Kityk et al. 2012; Qi et al. 2013). Normally, this tryptophan has emission peak around 342 nm in the presence of ADP or the absence of nucleotide. In the presence of ATP, the peak is shifted to

Fig. 7 Formation of the disulfide-bonded dimer of DnaK-C15A-A303C-H541C abolished chaperone activity. **a** Refolding of heat-denatured luciferase by DnaK and its co-chaperones DnaJ and GrpE. The activity of unheated luciferase was set as 100%. The names of DnaK proteins are the same as in Fig. 3. **b** Growth test of DnaK mutants. Serial dilutions of fresh cultures carrying indicated DnaK mutants were spotted on LB agar plates and incubated for 1 overnight at 30 and 35 °C

around 335 nm. The dimer was formed through two disulfide bonds between NBD from one protomer and SBD α from the other protomer. The observation that the peak of tryptophan fluorescence for the dimer was always locked around 335 nm even in the absence of ATP is consistent with the hypothesis that the disulfide bonds must have forced SBD α to form contacts with NBD within the same monomer as in the DnaK-ATP structure.

Forming these disulfide bonds in the dimer must have restricted the freedom of NBD-SBD β association although the disulfide bonds are far away from the NBD-SBD β interface. This is based on the observations that the kinetics of peptide substrate binding is amazingly fast even in the absence of the ATP, and addition of ATP does not induce significant release of bound peptide substrate. Normally, DnaK only has fast kinetics for peptide binding in the presence of ATP (Schmid et al. 1994). Due to the drastically reduced affinity in the ATP-bound state, ATP binding results in significant release of bound peptide substrate. Both properties are due to the specific NBD-SBD β contacts in the ATP-bound state as observed in the DnaK-ATP structures (Kityk et al. 2012; Qi et al. 2013). These contacts result in conformational changes of SBD β , especially around the peptide-binding pocket, which changes the affinity and kinetics of peptide substrate binding (Buchberger et al. 1995; Swain et al. 2007; Kityk et al. 2012; Qi et al. 2013; Yang et al. 2015; Zhuravleva and Gierasch 2015). The formation of the dimer must have forced the NBD-SBD β contacts as seen in the DnaK-ATP structure even in the absence of ATP although the disulfide bonds are formed between NBD and SBD α and far away from the NBD-SBD β interface. This indicates that the NBDs in the dimer are most likely restrained in the ATP-bound conformation even in the

absence of ATP. NBD is composed of two big lobes: I and II. Nucleotides, ATP or ADP, bind between the two lobes. The two lobes rotate against each other for more than 20° between the ATP- and ADP-bound states (Liu and Hendrickson 2007; Kityk et al. 2012; Qi et al. 2013). Only in the ATP-bound state the relative orientation of the two lobes of NBD provides suitable surfaces for SBD β to contact. The reduced ATP hydrolysis rate observed for the dimer is consistent with this hypothesis. It is possible that when the NBDs are forced to form to contact with SBD α through disulfide bonds as in our dimer, this changes the conformational landscape of DnaK and favors the NBD-SBD β interaction.

Both the reduced ATP hydrolysis rate and decreased affinity for peptide substrate observed for the dimer are consistent with the ATP-bound state. Moreover, the reduced ATPase rate of this dimer suggested that efficient ATP hydrolysis requires the dissociation of the DnaK dimer to progress through the chaperone cycle. Hsp70 has been shown to form a specific dimer in the ADP-bound state different from the DnaK-ATP dimer (Morgner et al. 2015). The dimer formed in the ATP-bound state must first dissociate to allow efficient ATP hydrolysis. After ATP is hydrolyzed into ADP, Hsp70 forms the dimer specifically for the ADP-bound state to allow productive interaction with Hsp90 (Morgner et al. 2015).

The most intriguing result is the Hsp40 interaction. Our previous biochemical analysis of a number of mutations on the dimer interfaces suggested that the dimer observed in the DnaK-ATP structure is essential for efficient interaction with Hsp40 co-chaperone DnaJ (Sarheng et al. 2015). This observation also indicated that the DnaK-ATP dimer may have enhanced interaction with DnaJ. However, restricting DnaK-ATP dimer using disulfide bonds in this study surprisingly showed

significant compromised interaction with DnaJ. This could be related to the reduced ATPase rate and decreased affinity for peptide substrate of the DnaK-A303C-H541C dimer. It was shown before that compromising either ATPase activity or the peptide-binding activity resulted in defects in the Hsp70-Hsp40 interaction (Suh et al. 1998, 1999; Laufen et al. 1999; Mayer et al. 1999). A possible explanation is that the stable DnaK-DnaJ interaction needs the dissociation of the DnaK-ATP dimer. The initial interaction between DnaK and DnaJ requires the formation of DnaK dimer. After this initial contact, to form a stable interaction to be detected by surface plasmon resonance, this dimer has to dissociate. ATP hydrolysis may be a natural way to break the dimer during the chaperone cycle. This could be why ATP hydrolysis was observed to be required for productive DnaK-DnaJ interaction (Wawrzynow and Zylicz 1995; Suh et al. 1998, 1999; Laufen et al. 1999; Mayer et al. 1999). The DnaK-DnaJ interaction is still largely a mystery even after years of extensive efforts. Besides the contacts between R167 of DnaK and D35 of DnaJ revealed by the breakthrough work from Gross's group (Suh et al. 1998), we know very little about the interface of this important interaction. A NMR study suggested that the interfaces of the DnaK-DnaJ interaction are dynamic in the presence of ADP although the functional DnaK-DnaJ interaction mainly occurs when DnaK binds ATP (Ahmad et al. 2011). A crystal structure of a DnaK-DnaJ complex will greatly advance our understanding on the molecular mechanism of the essential DnaK-DnaJ interaction and its role in protein folding.

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