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Detection of a concerted conformational change in the ATPase domain of DnaK triggered by peptide binding

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Abstract The molecular chaperone DnaK is composed of two functional domains, the ATPase domain and the substrate-binding domain. In this report, we show that peptide binding to DnaK can be sensed in real time through a labeled nucleotide bound in the ATPase domain. Specifically, when *N*8-(4-*N*′-methylanthraniloylaminobutyl)-8-aminoadenosine 5′-triphosphate (MABA)–ATP·DnaK complexes are rapidly mixed with excess peptide, MABA fluorescence rapidly increases and the rate of increase is proportional to peptide concentration. Analysis of the formation traces yield on and off rate constants that are exactly equal to the rate constants obtained from experiments that directly probe peptide binding to DnaK. These results are the first to show that peptide binding to ATP·DnaK triggers a concerted conformational change in the ATPase domain.

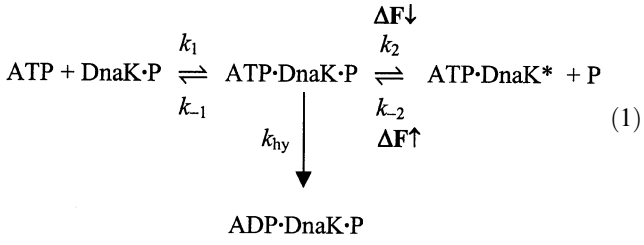
Key words: Chaperone; Allosteric; Concerted; Interdomain communication; *N*8-(4-*N*′-Methylanthraniloylaminobutyl)-8-aminoadenosine 5′-triphosphate

1. Introduction

The *Escherichia coli* molecular chaperone DnaK functions co-translationally and post-translationally to promote protein folding and to prevent and even reverse the formation of toxic protein aggregates. DnaK shuttles between an ATP-bound form, which has low affinity for peptide substrates, and an ADP-bound form, which has relatively high affinity for protein substrates. For reviews, see [1–4].

DnaK is composed of two functional domains: the N-terminal ATPase domain, which binds and hydrolyzes ATP [5], is comprised of residues 1–387, whereas the C-terminal substrate-binding domain, which binds and releases polypeptide targets [6,7], is comprised of residues 388–638. A helical lid, comprised of residues 507–638, encapsulates the bound peptide in the ADP-bound state. The activities of these two functional domains are coupled, i.e. ligand-induced conformational changes are transmitted in a reciprocal fashion between the two domains. For example, ATP binding triggers the rapid release of peptide substrates from the peptide-binding domain [8,9]. Conversely, peptide binding stimulates DnaK-mediated ATP hydrolysis [10–12]. It is noteworthy that other than these observations that peptide stimulates the steady-state hydrolysis of ATP by DnaK no evidence exists that peptide binding directly alters the conformation of the ATPase domain.

Insights into interdomain communication in DnaK have come about from kinetics studies. The kinetics of the interaction of ATP with DnaK are consistent with the following mechanism [13]



(1)

In the forward reaction, rapid ATP binding occurs in the first step, and a global conformational change occurs in the second step that ejects the bound peptide and simultaneously reduces the tryptophan fluorescence of the protein (denoted by the asterisk). The evidence for this mechanism is that the ATP-induced decrease in tryptophan fluorescence occurs in a single phase, and the observed first-order rate constant, k_{obs}, exhibits a hyperbolic dependence on ATP. Peptide release may also be followed directly using a labeled peptide [13,14]. Such rapid mixing experiments have shown that ATP induces peptide release at the same rate as the ATP-induced decrease in fluorescence ($k_{off} = k_{obs}$) [13]. It is intriguing that when a solution of peptide is mixed with ATP·DnaK* complexes peptide binding rapidly increases the protein's tryptophan fluorescence, consistent with the reversal of the second step of Eq. 1 [13,14]. ATP hydrolysis, we propose, occurs from the intermediate, ATP·DnaK·P.

In this study, we used a fluorescently tagged analog of ATP, *N*8-(4-*N*′-methylanthraniloylaminobutyl)-8-aminoadenosine 5′-triphosphate–ATP (MABA–ATP), to probe reverse reaction 1 in a novel way. MABA–ADP and –ATP have been used to probe the kinetics [15] and thermodynamics [16] of nucleotide binding to DnaK isolated from *E. coli* as well as from thermophilic bacteria [17]. This fluorescent derivative of ATP is the only one that successfully mimics ATP. For exam-

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Abbreviations: HEPES, *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid; acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene; Cro, the synthetic peptide MQERITLKDYAM; MABA, *N*8-(4-*N*′-methylanthraniloylaminobutyl)-8-aminoadenosine 5′-triphosphate; p5, the synthetic peptide CLLLSAPRR

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ple, ATP and MABA–ATP are hydrolyzed by DnaK with rate constants of $1.5(\pm 0.1)\times 10^{-3}$ s^{-1} and $1.6(\pm 0.1)\times 10^{-3}$ s^{-1} [15], respectively. Here we conducted rapid mixing experiments in which pre-formed MABA–ATP·DnaK complexes were mixed with unlabeled peptide and show that, remarkably, peptide binding to DnaK is sensed in real time via the MABA–ATP molecule bound in the ATPase domain.

2. Materials and methods

2.1. Protein and reagents

All reagents were of the highest purity and were purchased from Sigma, unless stated otherwise. DnaK was isolated as previously described [18] and maintained in the sample buffer (25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES)/50 mM KCl/5 mM $MgCl_2$/5 mM 2-mercaptoethanol at pH 7.0). Nucleotide was removed from wild-type DnaK either by exhaustive dialysis [19,20] or by the method of Gao et al. that employs AMP-*p*-nitrophenol [21]. Protein was stored in the HEPES sample buffering containing 10% glycerol at −80°C prior to use. MABA–ATP (>95% purity) was synthesized for us by TriLink BioTechnologies (San Diego, CA, USA). The p5 (CLLLSAPRR) [22] and Cro (MQERITLKDYAM) [18] peptides were purchased from Genemed Synthesis (South San Francisco, CA, USA), purified to >95% by high performance liquid chromatography, and peptide masses was verified by electrospray mass spectroscopy. The symbol '–' represents a covalent bond, such as found between the MABA group and ATP (MABA–ATP), and '·' represents a non-covalent interaction, such as MABA–ATP bound in the ATPase domain of DnaK (MABA–ATP·DnaK).

2.2. DnaK(1–388)

The pMSK plasmid harboring the wild-type *dnaK* gene behind an isopropyl-β-D-thiogalactose-inducible promoter was a gift to us from Dr. Lila Gierasch (University of Massachusetts, Amherst, MA, USA). Using the QuikChange Site-Directed Mutagenesis Kit (Stratagene), a stop codon was introduced into the *dnaK* gene such that translation terminates at residue 388. The forward and reverse primers were 5′-CTG ACT GGT GAC GTA AAA TAA GTA-CTG CTG CTG G-3′ and 5′-CCA GCA GCA GTA CTT ATT TTA CGT CAC CAG-TCA G-3′, respectively. To ensure that a secondary mutation was not introduced during the polymerase chain reaction protocol, the gene encoding DnaK(1–388) was sequenced at the Iowa State University DNA Sequencing and Synthesis Facility. The ATPase domain was expressed in the DnaK-deficient *E. coli* strain BB1553 [23] and purified and made nucleotide-free using the same methods as for the wild-type protein.

2.3. Stopped-flow fluorescence

A SX-18MV stopped-flow fluorescence spectrometer (Applied Photophysics, Leatherhead, UK) was used to monitor reverse reaction 1. The instrument's features have been described previously in detail [13,20]. Reverse reaction 1 was followed by mixing MABA–ATP·DnaK complexes with excess peptide and recording the changes in MABA fluorescence. The excitation wavelength was 340 ± 2 nm and an Oriel 418 nm cut-off filter was used to eliminate wavelengths less than 418 from impinging on the photomultiplier tube. Each stopped-flow trace is the average of four to 10 individual traces. Temperature control of both the jacketed reactants and the jacketed mixing chamber was achieved with a circulating external water bath ($\Delta T = \pm 0.2$°C). The concentrations in the text refer to after mixing.

2.4. Curve fitting

Stopped-flow data were fitted to single exponential function using a curve-fitting program that used a Marquardt algorithm based on the program Curfit given in Bevington [24]. Least squares fitting of data to hyperbolic or linear equations and determinations of standard errors of the fitted parameters were conducted using the program KaleidaGraph (Synergy Software, Reading, PA, USA).

3. Results

In the course of using the labeled compound MABA–ATP, we found that when MABA–ATP·DnaK complexes are mixed with excess unlabeled peptide a rapid burst of MABA fluorescence occurs. Intrigued by this finding, we analyzed the reaction in more detail. In the experiments described below, stopped-flow experiments were carried out by varying the concentration of peptide at a fixed concentration of MABA–ATP·DnaK complexes (3.5 μM).

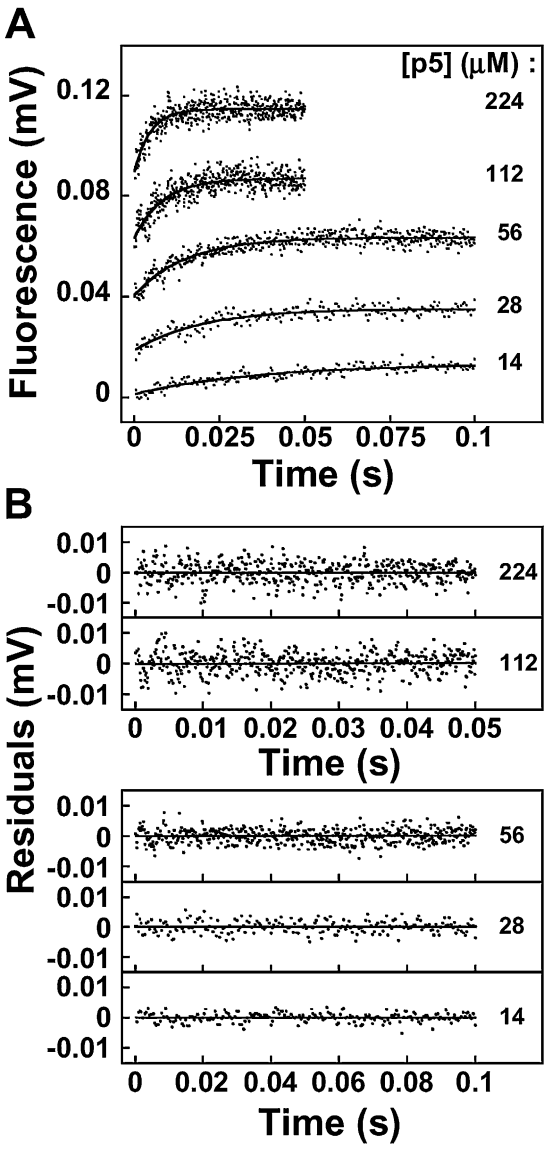

Fig. 1. A: Peptide binding enhances the fluorescence of MABA–ATP bound to DnaK. Traces were obtained upon mixing MABA–ATP·DnaK (3.5 μM) with increasing concentrations of the p5 peptide. Each trace follows single exponential kinetics, $F(t) = \Delta F(1-e^{-k_{obs}^{on}t}) + F_o$, where k_{obs}^{on}, ΔF, and F_o are the observed first-order rate constant, amplitude, and initial fluorescence, respectively (solid line). Values for k_{obs}^{on} are plotted against [peptide] in Fig. 3. B: Residuals (theoretical minus experimental). Temperature = 25°C.

Representative 'formation' traces are shown in Fig. 1A. We found that the rate and the amplitude of the MABA fluorescence signal increase with increasing concentration of the p5 peptide. Traces follow single exponential kinetics. Our interpretation is that peptide binding partially reverses reaction 1, according to:

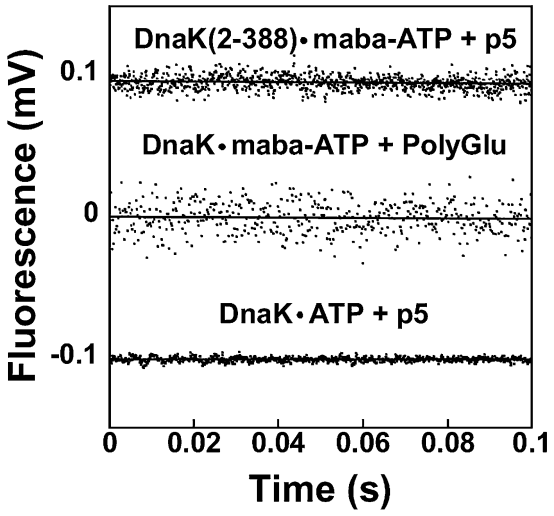

Fig. 2. Controls. The top trace shows the signal obtained upon mixing DnaK(2–388)·MABA–ATP complexes (3.5 μM) with excess p5 peptide (448 μM). The middle trace shows the signal obtained upon mixing DnaK·MABA–ATP complexes (3.5 μM) with polyglutamic acid (640 μM). The bottom trace shows the signal obtained upon mixing DnaK·ATP complexes (4 μM) with excess p5 peptide (448 μM). Temperature = 25°C.

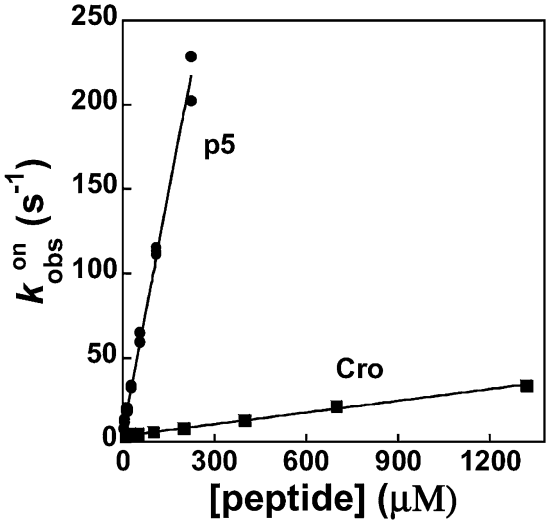

Fig. 3. Plots of k^{on}_{obs} versus [peptide]. Results are shown for two different peptides, p5 (●) and Cro (■). Solid lines are linear least square fits to the equation $k^{on}_{obs} = k_{on}[peptide] + k_{off}$. P5: $k_{on} = 9.4 \pm 0.2 \times 10^5$ M^{-1} s^{-1} and $k_{off} = 7.0 \pm 1.9$ s^{-1} ($R = 0.9968$). Cro: $k_{on} = 2.3 \pm 0.1 \times 10^4$ M^{-1} s^{-1} and $k_{off} = 3.5 \pm 0.2$ s^{-1} ($R = 0.9986$).

$$\text{MABA–ATP·DnaK}^* + \text{P} \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \text{MABA–ATP·DnaK·P} \qquad (2)$$

The asterisk in the above equation denotes the low-affinity state of the protein. MABA–ATP·DnaK* and MABA–ATP·DnaK·P represent two different conformations of DnaK.

We propose that a conformational change induced by peptide binding is transmitted from the C-terminal peptide-binding domain to the N-terminal ATPase domain via changes in the allosteric interface between the two domains, and this alters the conformation of the ATPase domain, which causes the increase in MABA fluorescence.

Several control experiments were conducted. In each experiment, as those above, $\lambda_{ex} = 340$ nm and $\lambda_{em} > 420$ nm. (i) Perhaps the p5 peptide binds to the MABA–ATP molecule lodged in the ATPase domain of DnaK, and this causes the increase in MABA fluorescence. Preformed complexes of MABA–ATP·DnaK(2–388), which is the ATPase domain of DnaK, were rapidly mixed with excess p5 peptide. No increase in MABA fluorescence is observed (Fig. 2, top trace). Thus, peptide binding directly to the MABA group lodged in the ATPase domain of DnaK is ruled out. (ii) If the peptide-induced increase in MABA fluorescence is truly peptide-specific, then a non-binder, such as polyglutamic acid, should have no effect on MABA fluorescence. Indeed, upon mixing MABA–ATP·DnaK complexes with excess polyglutamic acid, no change in MABA fluorescence is observed (Fig. 2, middle trace). This proves that the increase in MABA fluorescence is peptide-specific. (iii) We have reported that peptide binding to the ATP-bound state of DnaK results in a rapid increase in the tryptophan fluorescence of the protein. To rule out the possibility that the peptide-dependent increase in MABA fluorescence is linked in any way to tryptophan fluorescence, preformed ATP·DnaK* complexes were mixed with excess p5 peptide (Fig. 2, bottom trace). No change in fluorescence is observed upon mixing. This means that the peptide-induced increase in MABA fluorescence is unrelated to the presence of Trp102. (iv) The last control consisted of rapidly mixing MABA–ATP with excess peptide and monitoring for spectral changes in MABA fluorescence. This experiment produces no change in MABA fluorescence (data not shown). The com-

Table 1
Comparison of peptide on and off rate constants for the reaction

$$\text{ATP·DnaK}^* + \text{P} \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \text{ATP·DnaK·P}$$

Reaction (method)[a]	k_2 (s^{-1})	k_{-2} (M^{-1} s^{-1})
p5+MABA–ATP·DnaK (MABA fluorescence)	7.0 (±1.9)	9.4 (±0.2)×10^5
p5+ATP·DnaK (Trp fluorescence)[b]	7.0 (±3.1)	9.4 (±0.4)×10^5
ap5+ATP·DnaK (acrylodan fluorescence)[b]	4.5 (±1.3)	1.1 (±0.1)×10^6
Cro+MABA–ATP·DnaK (MABA fluorescence)	3.5 (±0.2)	2.3 (±0.1)×10^4
Cro+ATP·DnaK (Trp fluorescence)[c]	2.9 (±0.5)	2.4 (±0.4)×10^4

a, acrylodan.
[a]Rate constants were determined by following increases in either MABA fluorescence (of labeled ATP) or tryptophan fluorescence of DnaK, or acrylodan fluorescence (of the acrylodan-labeled peptide).
[b]Taken from [14].
[c]Taken from [13].

bined experiments show that the C-terminal domain of DnaK is required for the increase in MABA fluorescence, and the increase is peptide-specific. The MABA spectral changes are a consequence of interdomain communication.

The traces shown in Fig. 1 were obtained using the p5 peptide. Identical experiments were also conducted using the Cro peptide. The observed rate constants for the p5- and Cro-induced increase in MABA fluorescence are plotted against peptide concentration in Fig. 3. The linear nature of the plots is consistent with and expected for reaction 2. From the slope and *y*-intercept of the plots we obtain the on and off rate constants (Table 1). The 'on' and 'off' rate constants determined from these experiments exactly equal the rate constants determined from direct binding studies (a direct binding study is where the peptide is labeled with a fluorophore, and changes in the labeled peptide's fluorescence are monitored upon binding to DnaK [9,18]).

4. Discussion

This is the first evidence that peptide binding to ATP·DnaK* is instantaneously sensed within the ATPase domain. The results lead to the conclusion that interdomain communication in DnaK occurs via a concerted mechanism. Below we integrate this new information into the existing mechanism for reciprocal conformational changes in DnaK.

We know that reaction 1 explains the kinetics of ATP binding to both the wild-type protein and its various lidless variants. This means that interdomain coupling takes place without the lid, although it is greatly accelerated compared to the wild-type protein [14]. The role of the lid is to dramatically slow down the ATP-induced conformational change. Reversing reaction 1, viz. by mixing ATP·DnaK* complexes with peptide, increases the tryptophan fluorescence of the protein at a rate that is much greater than the rate of DnaK-mediated ATP hydrolysis [13]. This reaction is unique because the on and off rate constants obtained from these experiments exactly equal the rate constants obtained from direct peptide binding experiments (Table 1). But why is Trp102 such an excellent reporter for peptide binding to the low-affinity state of DnaK?

A recent study may shed light on why Trp102 fluorescence is an accurate reporter for peptide binding. Moro and colleagues [25] showed that the ATP-induced decrease in DnaK's tryptophan fluorescence is contingent upon the presence of the αA and 1/2 αB lid helices. Thus, for example, in the lidless variant DnaK(1–507), which lacks αA helix and all downstream residues, ATP induces the high-to-low affinity conformational change in the protein [26] but not the decrease in tryptophan fluorescence [25]. One idea is that ATP binding triggers movement of the αA and αB helices into the vicinity of Trp102, which is located on the outside of one of the lobes of the ATPase domain, and this changes the environment around Trp102, which causes quenching. Conversely, peptide binding to ATP·DnaK* reverses the position of the two lid helices, which relieves quenching. The spectral changes attributed to the conformational switch (second step of reaction 1) are probably due to these reversible changes in the environment around Trp102 at the domain–domain interface. Since interdomain communication occurs in the absence of the lid, producing no changes whatsoever in Trp102 fluorescence, the existence of these reversible spectral changes in the wild-type protein, or even in DnaK(2–517), may be thought of as a byproduct of the primary coupling mechanism. Thus, the peptide-induced increase in Trp102 fluorescence does not necessarily prove that peptide binding produces a conformational change within the ATPase domain.

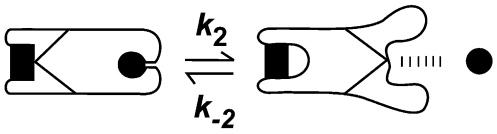

Fig. 4. Proposed conformational switch. This switch is the second step of two-step reaction 1. ATP and the substrate polypeptide are represented by the square and circle, respectively. The horizontal 'V' is the domain–domain interface that also contains the linker residues.

The present study is the first to show that peptide binding to DnaK triggers an instantaneous conformational change within the ATPase domain (of the ATP·DnaK·P intermediate). We infer this from the ability of peptide to trigger an increase in MABA fluorescence and because the rate constants derived from this assay exactly equal those obtained by quite disparate methods (Table 1). On the basis of this work, we propose that peptide binding to ATP·DnaK* triggers a concerted conformational change in the ATPase domain (Fig. 4). 'Concerted' means that the conformational change in the ATPase domain occurs simultaneously with the conformational change in the substrate-binding domain. It is likely that this peptide-triggered conformational change in the ATPase domain is key to the entire mechanism of DnaK because it links DnaK-mediated ATP hydrolysis with substrate binding; it enables DnaK to hydrolyze ATP more efficiently.

The ATPase and substrate-binding domains of DnaK probably contact each other over a substantial surface area. Additionally, the domains are connected via a highly conserved linker group of residues between the two domains. These contacts and connections enable perfect tight coupling between the two domains. Zero coupling between the two domains results in no communication whatsoever between the two domains. There have been several reports where mutations in the linker [27], or point mutations in other residues at the domain–domain interface [28,29], abolish domain–domain communication. In such a mutant, ATP binds and is hydrolyzed by the ATPase domain, but these two processes have no effect on substrate binding and release by the substrate-binding domain. It will be interesting to find point mutations in DnaK that reduce but do not eliminate the coupling between the two domains.

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