Mutations in the interdomain linker region of DnaK abolish the chaperone action of the DnaK/DnaJ/GrpE system

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Received 16 April 2001; accepted 24 April 2001

First published online 4 May 2001

Edited by Jesus Avila

Abstract Hsp70s assist the folding of proteins in an ATP-dependent manner. DnaK, the Hsp70 of *Escherichia coli*, acts in concert with its co-chaperones DnaJ and GrpE. Amino acid substitutions (D388R and L391S/L392G) in the linker region between the ATPase and substrate-binding domain did not affect the functional domain coupling and oligomerization of DnaK. The intrinsic ATPase activity was enhanced up to 10-fold. However, the ATPase activity of DnaK L391S/L392G, if stimulated by DnaJ plus protein substrate, was five times lower than that of wild-type DnaK and DnaK D388R. This defect correlated with the complete loss of chaperone action in luciferase refolding. Apparently, the conserved leucine residues in the linker mediate the synergistic effects of DnaJ and protein substrate on ATPase activity, a function which might be essential for chaperone action.

Key words: Molecular chaperone; DnaK; DnaJ; Hsp70; Hsp40; Oligomerization

1. Introduction

Molecular chaperones of the Hsp70 family bind to apolar segments of unfolded or only partially folded proteins and thus prevent their misfolding and aggregation. In addition, Hsp70s are essential for the translocation of proteins through membranes, as well as the assembly and disassembly of multiprotein structures [1]. Hsp70s comprise an invariant NH_2-terminal 44-kDa ATPase domain followed by a rather variant 15-kDa peptide-binding domain and a 10-kDa COOH-terminal region of unknown function. The structures of the two separate domains have been determined by X-ray crystallography [2,3]. The domains are connected by a highly conserved segment of 13 amino acid residues. Interdomain communication is an integral part of the mechanism of action of Hsp70s [4–8].

DnaK, the Hsp70 of *Escherichia coli*, functions together with its co-chaperones DnaJ and GrpE. DnaK alternates between the ATP-liganded state, characterized by low affinity for target polypeptides and fast rates of binding and release, and the ADP-liganded state with high affinity and low exchange rates for substrates [4,9]. DnaJ triggers the conversion of the high-affinity state to the low-affinity state by stimulating the hydrolysis of DnaK-bound ATP, and, moreover, is supposed to target the substrate to DnaK [10–13]. A recent nuclear magnetic resonance study localized the binding site for DnaJ to the ATPase domain of DnaK [14]. However, there is evidence that DnaJ also binds to the substrate-binding site of DnaK itself or in close proximity to this site [15,16]. GrpE, the second co-chaperone, controls the chaperone function of DnaK by accelerating the exchange of DnaK-bound ADP with ATP and thus completing the chaperone cycle. The structure of dimeric GrpE co-crystallized with the ATPase domain of DnaK has been determined [17].

In this study, we prepared two variants of DnaK with substitutions (D388R and L391S/L392G) in the highly conserved segment connecting the ATPase domain and the peptide-binding domain. The results indicate that the leucine triplet in the linker is essential for the joint control of the chaperone action by DnaJ and the protein substrate.

2. Materials and methods

2.1. Site-directed mutagenesis

For site-directed mutagenesis of DnaK in the region bp 1162–1172 (amino acid residues 389–392), the pTTQ19 vector containing the *dnak* gene (a gift from C. Georgopoulos and D. Ang, Geneva), was used as template for in vitro mutagenesis with the QuickChange Site-Directed Mutagenesis kit from Stratagene. Plasmid pTPG9 was amplified by polymerase chain reaction, using the following complementary primers: A_1: 5′-CTGACTGGTGACGTCAAA**CGC**GTACTG-CTGCTG-3′,A_2: 5′-CAGCAGCAGAC**GCG**TTTGACGCACCAG-TCAG-3′; B_1: 5′-GACGTCAAAGACGTA**TCGGGG**CTGGACGT-TACCCCG-3′, B_2: 5′-CGGGGTAACGTCCAG**CCCCGA**TACGT-CTTTTACGTC-3′. The exchanged nucleotides are underlined. The corresponding mutant chaperones are denoted as D388R and L391S/L392G, respectively. The positive clones were verified by sequencing (SequiTherm Excel Long-Read Cycle Sequencing kit-LC, Epicentre Technologies) with fluorescent primers using a DNA sequencer (LI-COR).

2.2. Expression and purification of proteins

The wild-type (wt) and mutant forms of DnaK were overexpressed by isopropyl-β-D-1-thiogalactopyranoside induction of pTPQ9 in *E. coli* BB1553 cells [18]. The expressed proteins were purified as described previously [8,9]. Their molecular masses were confirmed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry. The preparations of mutant and wt DnaK contained less than 0.1 mol of ADP per mol [8]. The proteins were stored as stock solutions in assay buffer (25 mM HEPES/NaOH, 100 mM KCl, 5 mM $MgCl_2$, pH 8.0) at −80°C. DnaJ and GrpE, prepared as reported previously [19,20], were a gift from H.-J. Schönfeld, Basel. The stock solutions were kept at −80°C in 50 mM Tris–HCl, 100 mM NaCl, pH 7.7.

2.3. Measurements of peptide binding and release

Peptide NR (NRLLLTG) and σ^{32} (QRKLFFNLRKTKQ) were

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PII: S0014-5793(01)02435-8

synthesized by Chiron Technologies and labeled with the environmentally sensitive fluorophore acrylodan (6-acrylolyl-2-dimethylaminonaphthalene, from Molecular Probes) and purified as described [8]. Fluorescence measurements of binding and release of the labeled peptides were performed in assay buffer as described [21]. The data were analyzed by using the program Sigmaplot 5.0 (from SPSS).

2.4. ATPase activity assays

Steady-state and single-turnover ATPase activity were determined as described previously [22,23]. [2,5′,8-^{3}H]ATP was obtained from Amersham Pharmacia Biotech. The data were analyzed with Sigmaplot 5.0.

2.5. Refolding of denatured luciferase

Luciferase of *Photinus pyralis* was purchased from Roche. Lyophilized material (1 mg) was dissolved in 1.66 ml of refolding buffer (50 mM Tris–HCl, 55 mM KCl, 5.5 mM dithiothreitol, pH 7.7), 0.2-μl filtrated and precipitated by addition of five volumes of acetone (−20°C, 30 min). After centrifugation for 10 min at 1000×*g* and 4°C, the pellet was re-dissolved in 1.66 ml denaturing buffer (6 M guanidine–HCl, 100 mM Tris–HCl, 10 mM 1,4-dithio-DL-threitol, pH 7.7) and filtrated through a 0.2-μm membrane. The refolding reaction was started by addition of 2 μl denatured luciferase (0.1 μM) to refolding buffer with a final dilution factor of 1:100. Luciferase activity was measured every 20 min for 2 h with a luminometer (Berthold, Switzerland). For details, see the legend of Fig. 2.

2.6. Size exclusion chromatography

For assessing the spontaneous oligomerization of DnaK, a Superdex 200 column (3.2/300 mm, fitted with a Smart system, from Pharmacia) was equilibrated with assay buffer with or without 2 mM ATP. DnaK (10 μM) was incubated for 30 min at room temperature. A 50-μl sample was loaded into the sample loop 1 min before injection. For DnaJ-triggered oligomerization of DnaK, the column was equilibrated with assay buffer containing 1 μM DnaJ and 2 mM ATP. DnaK (10 μM) was incubated in assay buffer containing 1 μM DnaJ and 2 mM ATP for 30 min at room temperature before being injected. The chromatography was performed with a flow rate of 100 μl/min at 25°C and monitored at 280 nm.

3. Results and discussion

We produced two variants of DnaK by amino acid substitutions in the linker segment connecting the ATPase domain and the substrate-binding domain of DnaK (387–394; **KDVLLLDV**, the amino acid residues in bold being highly conserved in the Hsp70 family). We exchanged either one of the conserved aspartate residues with an arginine residue (D388R) or replaced two of the three conserved leucine residues with a serine and a glycine residue (L391S/L392G). The peptide-binding properties of the mutant proteins were examined with the fluorescence-labeled peptide a-NR (acrylodan-NRLLLTG). The kinetic measurements (Fig. 1A) showed that the rates of complex formation of DnaK D388R and DnaK L391S/L392G with this target peptide are slightly faster and slower than that of wtDnaK, respectively (Table 1). The binding affinity of DnaK L391S/L392G for peptide a-NR as determined by titration (Fig. 1B, Table 1) is 3–4 times lower than that of wtDnaK and DnaK D388R. These relatively

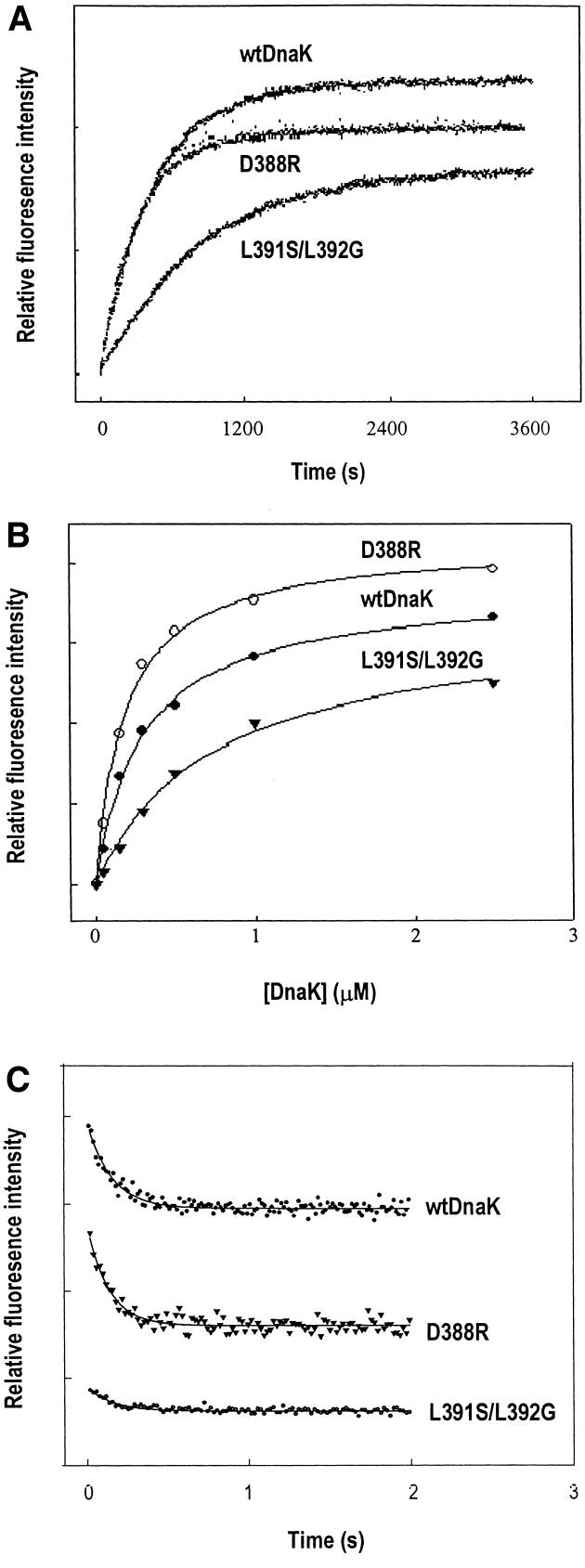

Fig. 1. Peptide binding to and release from wt and mutant DnaK. A: Kinetics of peptide binding in the absence of ATP. Peptide a-NR (50 nM) was added to DnaK (1 μM) at 25°C. B: Determination of the dissociation equilibrium constant (K_d) of the DnaK–peptide complex. a-NR (50 nM) was titrated with increasing concentrations of DnaK and the mutant chaperones (25 nM–2.5 μM) at 25°C. The amplitudes of the reactions in A and B were in fact similar but were set apart in the figure for clarity's sake. C: ATP-induced dissociation of DnaK–a-NR complex. a-NR (50 nM) was incubated with the chaperone DnaK (1 μM) at 25°C for 1 h. The reaction was triggered by the addition of 2 mM ATP in assay buffer. All reactions followed single exponential functions. The curves are arbitrarily set apart in the figure for clarity's sake. For details, see Section 2.

Table 1
Peptide binding to and release from wt and mutant DnaK

	k_{obs}[a] of complex formation (s^{-1})	K_d[b] (μM)	k_{obs}[c] of release (s^{-1})
wtDnaK	0.002	0.3	7.6
D389R	0.003	0.2	6.6
L391S/L392G	0.001	0.8	6.4

[a]Observed rate constant of complex formation of DnaK with a-NR in the absence of ATP as calculated from the data of Fig. 1A.
[b]Dissociation equilibrium constant of the DnaK–a-NR complex in the absence of ATP as calculated from the data of Fig. 1B.
[c]Observed rate constant of peptide release upon addition of 2 mM ATP to nucleotide-free DnaK–peptide complex as calculated from the data of Fig. 1C.

moderate differences between the mutant chaperones and wtDnaK in kinetic properties and binding affinity are in line with the crystal structure of the substrate-binding domain of DnaK [3] in which the linker is in no way part of the peptide-binding site.

Binding of ATP has been found to trigger the release of protein and peptide substrates from DnaK [4,9,24]. When ATP is added to the preformed complex of the chaperone with peptide a-NR, the DnaK mutants show virtually the same rate of peptide release as wtDnaK (Fig. 1C; Table 1). Release experiments with acrylodan-labeled σ^{32} peptide (a-QRKLFFNLRKTKQ) gave similar results (data not shown). Apparently, the mutations in the linker region do not affect the interdomain communication in DnaK.

Both DnaK D388R and DnaK L391S/L392G possessed higher intrinsic steady-state and single-turnover ATPase activities than wtDnaK (Table 2). The mutations in DnaK that have been reported previously to result in elevated intrinsic ATPase activity [25–29] were located in either the ATPase or the substrate-binding domain. Apparently, amino acid substitutions throughout the entire chaperone molecule result in enhanced intrinsic ATPase activity. It seems that in wtDnaK the intrinsic ATPase activity is suppressed to avoid futile consumption of ATP, and that the responsible suppression mechanism is impaired by almost any structural change in the chaperone molecule. Interactions of DnaK with the co-chaperone DnaJ and target polypeptides are known to stimulate the hydrolysis of DnaK-bound ATP [25,30]. The steady-state activities of the two DnaK mutants when stimulated by DnaJ plus GrpE and by peptide NR plus GrpE are in the same range as those of the wtDnaK though the intrinsic ATPase activity of the mutant proteins is considerably higher (Table 2).

The chaperone activities of the DnaK mutants were examined with guanidine–HCl-unfolded firefly luciferase as substrate. Efficient refolding of luciferase upon dilution of the denaturant requires the presence of DnaK, DnaJ, GrpE and ATP. DnaK L391S/L392G almost completely lacked chaperone activity in this assay while DnaK D388R showed an only slightly reduced yield of refolding (Fig. 2).

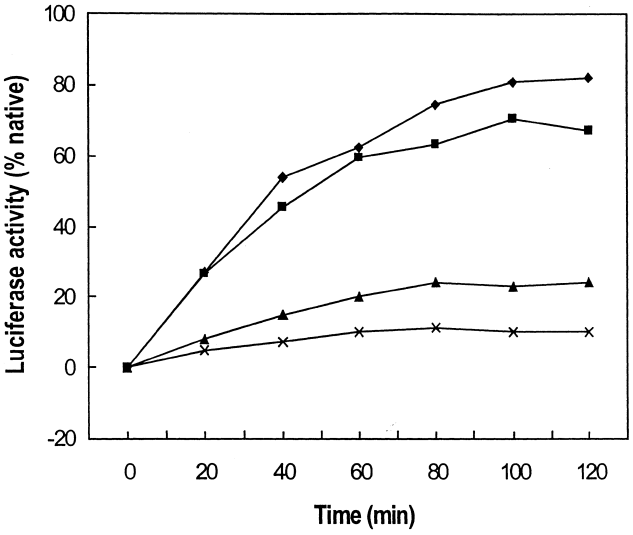

Fig. 2. Chaperone activity of DnaK mutants in luciferase refolding. For refolding, 1 μM DnaK, 0.1 μM DnaJ, 0.5 μM GrpE, 2 mM ATP and 15 mM $MgCl_2$ were mixed and the total protein concentration was set to 0.5 mg/ml by supplementation with bovine serum albumin. Guanidine–HCl denatured luciferase (1 μM; for details of the denaturation protocol, see Section 2) was diluted into the above refolding reaction mixture as described in Section 2. WtDnaK (♦); DnaK D388R (■); DnaK L391S/L392G (▲); denatured luciferase without chaperone (×) was used as control.

Neither mutation significantly affects the interdomain communication as shown by the unchanged effect of peptide binding and release (Table 1) as well as by the partially maintained stimulatory effects of peptide and denatured protein substrate on ATPase activity (Table 2). The loss of luciferase refolding activity of DnaK L391S/L392G might thus be ascribed to impaired co-operation between DnaK and the co-chaperones. Indeed, the single-turnover ATPase activity of DnaK L391S/L392G when stimulated conjointly by DnaJ and denatured luciferase is five times lower than the activities of wtDnaK and DnaK D388R under the same conditions (Table 2). This loss of the synergistic effects of DnaJ and denatured luciferase in DnaK L391S/L392G suggests that the leucine triplet in the linker segment is essential for combining the stim-

Table 2
Steady-state and single-turnover ATPase activities

	Steady-state ATPase activity (min^{-1})			Single-turnover ATPase activity (min^{-1})			
	intrinsic	+DnaJ+GrpE	+GrpE+NR	intrinsic	+DnaJ	+denatured luciferase	+DnaJ+denatured luciferase
DnaK	0.075	4.95	0.15	0.02	0.24	0.042	2.3
D388R	0.15	1.5	0.3	0.06	0.48	0.11	1.8
L391S/L392G	0.75	1.5	0.9	0.1	0.32	0.14	0.5

Steady-state ATPase activity of wtDnaK and mutant DnaK was determined in 50-μl reaction mixtures at 25°C. DnaK (10 μM), DnaJ (1 μM) and GrpE (0.5 μM), and peptide NR (50 μM; in the assay for peptide-stimulated ATPase activity) were pre-incubated in assay buffer for 30 min. The reaction was triggered by the addition of ATP (2 mM final concentration). For determination of single-turnover ATPase activity, DnaK–ATP complexes were formed by incubating DnaK with ATP for 15 min at 25°C and isolated by rapid size-exclusion chromatography at low temperature. The final concentrations of DnaK–ATP complex and DnaJ were adjusted to 1 and 0.1 μM, respectively. Luciferase was denatured in 3 M guanidine–HCl and diluted into refolding buffer. The final concentration of denatured luciferase was 2 μM; the final concentration of guanidine–HCl was 150 mM and did not affect the ATPase activity of DnaK.

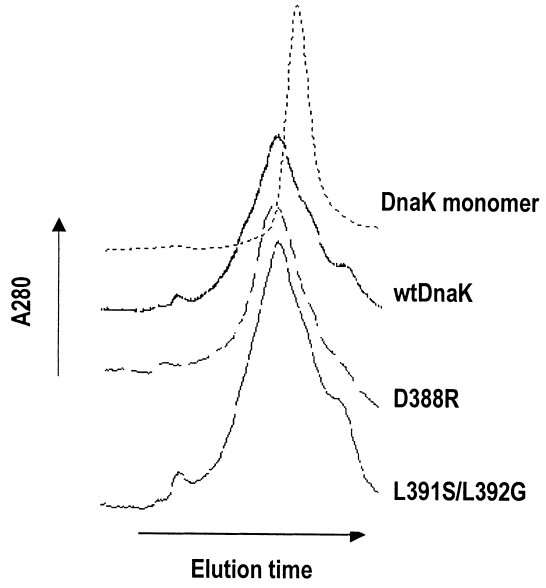

Fig. 3. Oligomerization of wtDnaK and DnaK mutants in the presence of DnaJ and ATP. WtDnaK monomer formed in the presence of ATP was taken as control. The Superdex 200 column was equilibrated with assay buffer containing 2 mM ATP and 1 μM of DnaJ (for details, see Section 2). DnaK and mutant proteins (10 μM) were incubated with 2 mM ATP and 1 μM DnaJ for 30 min at room temperature, respectively, before the injection. Monomeric DnaK was eluted at 16 min and the oligomers at 14 min.

ulatory effects of the two different ligands of DnaK, a function that perhaps might relate with the substrate targeting function of DnaJ and the loss of which might underlie the abolished chaperone effect of DnaK L391S/L392G (Fig. 2).

Members of the Hsp70 family such as DnaK, BiP and Hsc70 have been reported to self-associate to form oligomeric species [31,20,32]. Apparently, self-association is a general feature of the Hsp70 family that might be of mechanistic importance. Nucleotide-free or ADP-liganded DnaK exists as a monomer in equilibrium with oligomers of different size. In the presence of ATP, DnaK exists exclusively as a monomer; however, if DnaJ is added to DnaK in the presence of ATP, self-association of DnaK occurs. The DnaJ-triggered oligomerization was suggested to reflect the ability of DnaJ to present one DnaK molecule to another, just like targeting protein substrates to DnaK [33]. We investigated the linker mutants for their propensity to oligomerize. Size-exclusion chromatography in the absence of ATP showed both mutants to oligomerize spontaneously in a fashion very similar to that found with wtDnaK (not shown). For examination of DnaJ-triggered oligomerization, the column was equilibrated with ATP and DnaJ. Under the tested conditions, both mutant proteins formed oligomers in the presence of DnaJ and ATP quite similar to wtDnaK (Fig. 3). The present finding, that L391S/L392G is not effective in refolding denatured luciferase, but still, as wtDnaK does, self-associates to oligomers in the presence of DnaJ and ATP, suggests that oligomerization of DnaK in the presence of DnaJ and ATP is different from the DnaJ-controlled interaction of DnaK with macromolecular substrates.

In summary, amino acid substitutions in the interdomain linker region of DnaK indicate an important, yet to be explored, role of the conserved leucine triplet in the interdomain linker for the interaction of DnaK (Hsp70) and DnaJ (Hsp40) with macromolecular substrates.

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