Functional Defects of the DnaK756 Mutant Chaperone of Escherichia coli Indicate Distinct Roles for Amino- and Carboxy-terminal Residues in Substrate and Co-chaperone Interaction and Interdomain Communication*

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The first discovery of an Hsp70 chaperone gene was the isolation of an Escherichia coli mutant, dnaK756, which rendered the cells resistant to lytic infection with bacteriophage λ. The DnaK756 mutant protein has since been used to establish many of the cellular roles and biochemical properties of DnaK. DnaK756 has three glycine-to-aspartate substitutions at residues 32, 455, and 468, which were reported to result in defects in intrinsic and GrpE-stimulated ATPase activities, substrate binding, stability of the substrate-binding domain, interdomain communication, and, consequently, defects in chaperone activity. To dissect the effects of the different amino acid substitutions in DnaK756, we analyzed two DnaK variants carrying only the amino-terminal (residue 32) or the two carboxy-terminal (residues 455 and 468) substitutions. The amino-terminal substitution interfered with the GrpE-stimulated ATPase activity. The carboxy-terminal mutations (i) affected stability and function of the substrate-binding domain, (ii) caused a 10-fold elevated ATP hydrolysis rate, but (iii) did not severely affect domain coupling. Surprisingly, DnaK chaperone activity was more severely compromised by the amino-terminal than by the carboxy-terminal amino acid substitutions both in vivo and in vitro. In the in vitro refolding of denatured firefly luciferase, the defect of the DnaK variant carrying the amino-terminal substitution resulted from its inability to release, upon GrpE-mediated nucleotide exchange, bound luciferase in a folding competent state. Our results indicate that the DnaK-DnaJ-GrpE chaperone system can tolerate suboptimal substrate binding, whereas the tight kinetic control of substrate dissociation by GrpE is essential.

In 1977, Georgopoulos (1) reported a mutation, groPC756

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later termed dnaK756, which conferred resistance to Escherichia coli against lytic infection by bacteriophage λ (2, 3). This was the first discovery of a member of the ubiquitous Hsp70 chaperone family (4–6), the DnaK heat shock protein, and the DnaK756 mutant protein has since been widely used to dissect cellular functions and biochemical features of DnaK. DnaK is now known to play central roles in assisting folding processes in the entire life span of proteins in the E. coli cytosol (7). In the present study we dissected the functional defects of the classical DnaK756 mutant protein, which provided insights into mechanistic features of DnaK.

DnaK consists of an amino-terminal, highly conserved ATPase domain (residues 1–385), an adjacent, conserved substrate-binding domain (residues 393–537), and a more diverse carboxy-terminal domain (residues 538–638) of unclear function. Substrate interactions of the substrate-binding domain are controlled by the nucleotide state of the ATPase domain, thus indicating functional coupling of the two domains (8). The ATP state is characterized by low affinity and fast exchange rates for substrates, whereas the ADP state possesses high affinity and low exchange rates for substrates (9, 10). ATP hydrolysis is a key step for DnaK chaperone function because it allows the locking-in of substrates in a stable complex with DnaK-ADP. ATP hydrolysis is a slow and rate-limiting step of the ATPase cycle (10–13), and most physiological substrates require the DnaJ co-chaperone or a DnaJ homologue for efficient binding to DnaK. DnaJ stimulates ATP hydrolysis by at least several hundred-fold (11, 14–19) provided that protein substrates are associated with the DnaK substrate-binding cavity (17, 18, 20). DnaJ interacts via its conserved amino-terminal J domain with the ATPase domain of DnaK (21–23) and in a coupled process appears to transfer substrates by additional interactions with the DnaK substrate-binding domain (5, 18). A second co-chaperone that is essential for most functions of DnaK is the GrpE nucleotide exchange factor. GrpE accelerates dissociation of DnaK-ADP-P, complexes by 3 orders of magnitude (24), thus allowing rapid rebinding of ATP to DnaK, which in turn triggers substrate release from DnaK.

The ATPase domain of DnaK (and of other Hsp70 proteins) consists of two subdomains separated by a deep central cleft (25–27, Fig. 7A). Two crossed α-helices connect the two subdomains and divide the cleft into an upper cleft, at the bottom of which nucleotide and Mg2+ are bound (26, 28), and a lower cleft constituting a potential DnaJ interaction site (22, 23). A conserved loop in subdomain Ia (residues 28–33) is essential for stable binding of GrpE in the absence of nucleotide (29). The substrate-binding domain is built by a compact β-sandwich
composed of eight antiparallel β-strands and two α-helices packed onto the β-sandwich (30, Fig. 7B). The upper strands of the β-sandwich are connected by two pairs of loops protruding upwards. Peptide substrates are tightly bound in a hydrophobic cavity formed by the inner loops and covered by the second, lid-like α-helix without any direct contacts.

The DnaK756 mutant protein has three glycine-to-aspargine substitutions, one at residue 32 in the GrpE-binding loop of the ATPase domain and two at residues 455 and 468 in strand 5 and loop 5,6, respectively, of the β-sandwich that forms the substrate-binding site (29, 31). It is unknown to what extent these alterations are contributing to the phenotypes of DnaK756. Defects of these alterations are contributing to the phenotypes of DnaK756, DnaK-G32D, and DnaK-G455D,G468D. In an earlier study, we generated two DnaK variants carrying the amino-terminal (DnaK-G32D) or the two carboxyl-terminal (DnaK-G455D,G468D) mutations and demonstrated that the G32D substitution is solely responsible for the reduced affinity of DnaK756 to GrpE in absence of nucleotide (29). Here, we report a detailed biochemical analysis of the DnaK756, DnaK-G32D, and DnaK-G455D,G468D mutant proteins.

EXPERIMENTAL PROCEDURES

Reagents—Unless indicated otherwise, all chemicals were of the highest purity available. AT.P-agarose (A 2767) and luciferase (L 9506) were from Sigma. Luciferin was from Fluka.

Peptides—Peptide ω32-Q132-Q144-C (QRKLFFNLRLRTKQC) (44) was chemically synthesized and high pressure liquid chromatography purified by Dr. Rainer Frank (Zentrum für Molekulare Biologie, Heidelberg, University Heidelberg). Peptide concentrations were determined by quantitative amino acid analysis. IANNS-conjugated (44) and 14C-NEM-labeled peptide ω32-Q132-Q144-C was kindly provided by Dr. J. McCarty.

Protein Purification—DnaK756 and its two derivatives, dnak-G32D and dnak-G455D,G468D, were cloned into an expression vector as described (29), and the DnaK proteins were expressed and purified in ΔdnaK52 cells as described (45). To isolate the possibility of homologous recombination between the plasmid-encoded dnaK allele and the (promoterless) truncated chromosomal dnaK gene of ΔdnaK52 cells, DnaK756 and DnaK-G455D,G468D were purified from ΔdnaK52 recA cells. The principal purification steps are ammonium sulfate precipitation of the DnaK756, DnaK-G32D, and DnaK-G455D,G468D mutant strains include resistance against bacteriophage λ (1), temperature-sensitive growth above 40 °C (3, 32), and defects in regulation of the heat shock response (32–35) and in cellular proteolysis (36–38). In addition, from in vitro studies it appears that the DnaK756 mutant protein possesses an altered ATPase activity (14, 39), reduced affinities for GrpE (29, 40) and substrates (41, 42), reduced interdomain coupling potential (42), as well as reduced thermostability and altered secondary structure (43). Despite this wealth of genetic and biochemical data, only little effort has been made to systematically analyze DnaK756 in vitro and to dissect the effects of the G32D substitution in the ATPase domain from the G545D and G468D substitutions in the substrate-binding domain. In an earlier study, we generated two DnaK variants carrying the amino-terminal (DnaK-G32D) or the two carboxyl-terminal (DnaK-G455D,G468D) mutations and demonstrated that the G32D substitution is solely responsible for the reduced affinity of DnaK756 to GrpE in absence of nucleotide (29). Here, we report a detailed biochemical analysis of the DnaK756, DnaK-G32D, and DnaK-G455D,G468D mutant proteins.

E. coli replication proteins were purified to near homogeneity as described previously (48–50).

In Vivo Phenotypes—The ability of wild type and mutant dnaK alleles to complement the ts and λ' phenotypes of ΔdnaK52 cells were determined as described (45).

λ in Vitro Replication Assay—Replication of ori λ plasmid DNA in vitro was performed essentially as described (48). The reaction mixture (30 µl) contained 40 mM Hepes/KOH, pH 7.6, 100 mM potassium glutamate, 11 mM magnesium acetate, 4 mM ATP, 180 µM each dATP, dCTP, and dGTP, 80 µM [3H]dATP (2.4 µCi/µmol of total deoxynucleotide), 50 µg/ml bovine serum albumin, 215 ng of ori λ-carrying pRLM4 DNA, 195 ng of λ O, 100 ng of λ P, 175 ng of DnaJ, 540 ng of SSB, 50 ng of DnaL 100 ng of primase, 80 ng of DNA polymerase III holoenzyme, 230 ng of GyrA, 240 ng of GyrB, and GrpE, mutant, or wild type DnaK proteins in amounts as indicated. The reaction mixture was incubated for 40 min at 30 °C followed by liquid scintillation counting of trichloroacetic acid-precipitable radiolabeled material.

Luciferase Refolding—Refolding of firefly luciferase denatured by heat (51) or guanidine hydrochloride (52) was analyzed exactly as described. The final protein concentrations in the refolding reactions were 80 nM luciferase, 160 nM DnaJ, 1200 nM DnaK, and 600 nM GrpE (heat denaturation) or 80 nM luciferase, 160 nM DnaJ, 800 nM DnaK, and 800 nM GrpE (chemical denaturation). Luciferase aggregation was monitored by right angle light scattering (51) as follows: Luciferase (10 µM) was chemically denatured for 30 min at 37 °C and then diluted in refolding buffer containing DnaK (1.6 µM) and DnaJ (80 nM). Aggregation was measured at 30 °C, and the excitation/ emission wavelength was set to 320 nm. In the experiment described in Fig. 6B and C, GrpE (final concentration, 800 nM) was added after 35 min to a standard aggregation assay. The reaction mixture was split, and subsequent aggregation and reactivation of luciferase were followed in parallel. The final concentrations in this reaction were: 160 nM luciferase, 1.6 µM DnaK, 80 nM DnaJ, and 800 nM GrpE.

ATPase Activity Measurements—ATPase activities under steady state conditions were determined as described (11, 45). Final concentrations were 200 µM ATP, 0.69 µM DnaK, 1.38 µM GrpE, and GrpE as indicated. ATP hydrolysis rates under single turnover conditions were determined as described (11, 22). Final concentrations were 1.2 µM DnaK ATP complex, 5 µM DnaJ, and 83 µM peptide ω32-Q132-Q144-C.

Statistics—The values given in Table I represent the average of n = 2–4 individual experiments and are given in the format ±, where the χ is the weighted mean value and the standard error of the weighted mean, and are calculated according to standard equations.

Peptide Binding—The dissociation constant (Kd) of DnaK for IAANS-conjugated peptide ω32-Q132-Q144-C was determined as described (44) by titration of DnaK at concentrations between 0.025 and 7.5 µM in the presence of 0.2 µM peptide. The fluorescence data were fitted by nonlinear regression to the equation

\[
F_0 = \frac{2}{K_d + [E]_0} \left( \frac{[E]_0 + K_d}{[E]_0} \right) \times (F_{\text{max}} - F_0) + F_0
\]

where \(F_0\) indicates measured fluorescence signal, \(E_0\) indicates DnaK concentration, \(L_0\) indicates peptide concentration, \(F_{\text{max}}\) indicates fluorescence amplitude, and \(F_{\text{off}}\) indicates intrinsic fluorescence in absence of DnaK. The equation was kindly provided by Dr. J. Reinstein (Max-Planck-Institut für Molekulare Physiologie, Dortmund, Germany). The apparent association rate constant \(k_{on}\) was calculated from the mean fluorescent values for \(K_d\) and \(k_{st}\) according to \(k_{on} = \frac{F_{\text{off}} - F_{\text{on}}}{[E]_0 \times (F_{\text{on}} - F_{\text{off}})}\). The combined standard error \(\alpha_{on}\) was calculated according to

\[
\frac{\alpha_{on}}{k_{on}} = \sqrt{\frac{\alpha_{off}}{F_{\text{off}}^2} + \frac{\alpha_{on}}{F_{\text{on}}^2}}
\]

where \(\alpha_{on}\) indicates \(\alpha_{on}\), \(\alpha_{off}\), and \(\alpha_{on}\) indicate weighted mean values for \(k_{st}\) and \(K_d\), and \(\alpha_{on}\), \(\alpha_{off}\), and \(\alpha_{on}\) indicate standard errors of \(F_{\text{off}}\) and \(F_{\text{on}}\).

Peptide Release—Release of DnaK-bound peptide by ATP was determined as described (11, 45). Final concentrations in the preincubation mixture were 5 µM DnaK and 5 µM 14C-labeled peptide ω32-Q132-Q144-C (0.57 Bq/µmol). Immediately prior to separation on a G50 nickel column (Amersham Pharmacia Biotech) at 4 °C, excess ATP (if indicated; final concentration, 1 mM) and unlabeled chase peptide (final concentration, 80 µM) were added. The extent of peptide release was expressed as the ratio of radioactivity present in the DnaK-peptide peak.
fractions in the absence versus presence of ATP.

Partial Proteolysis—Partial proteolysis by papain was performed at 30 °C in reaction mixtures (30 μl) containing 40 mM Hepes/KOH, pH 7.6, 1 mM Mg(OAc)₂, 2 mM dithiothreitol, 30 μg of DnaK. The reactions were started by addition of papain (Merck; final concentration, 10 μg/ml) that had been activated as described (53). E64 (Merck; final concentration, 1 mM) was added to aliquots taken at specified time points to irreversibly inhibit papain. Samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by silver staining (54).

RESULTS

ATPase Activity

Earlier studies on the steady state ATPase activity of DnaK756 were conflicting because they reported reduced (39) and 50-fold increased (14) ATPase activities. To determine more precisely the $k_{cat}$ of ATP hydrolysis by highly purified DnaK756, we performed single turnover ATPase measurements on isolated DnaK-ATP complexes (11, 22), thereby circumventing the potential problem of contaminating ATPases (Table I). The $k_{cat}$ for wild type DnaK was 0.038 min⁻¹, consistent with recent studies using similar assay conditions (0.02–0.1 min⁻¹; Refs. 10–13). This rate was identical to the $V_{max}$ of this DnaK preparation under steady state conditions (data not shown), indicating that ATP hydrolysis is the rate-limiting step of the unstimulated ATPase activity (10–13). The DnaK756 mutant protein had 10.3-fold increased $k_{cat}$ (Table I) and $V_{max}$ (not shown) values, thus unambiguously revealing alterations in the ATPase activity. Furthermore, the identical values for $k_{cat}$ and $V_{max}$ show that ATP hydrolysis is rate-limiting for the DnaK756 ATPase activity as well. Analysis of the DnaK-G32D and DnaK-G455D,G468D variants demonstrated that the increased $k_{cat}$ segregates with the carboxy-terminal mutations (12.6-fold elevated), whereas the aminoterminal mutation does not affect the $k_{cat}$ (Table I).

We then tested in single turnover assays the potentials of a high affinity peptide substrate for DnaK, ε⁶⁵⁻Q3596-Q444-C (44), and of DnaK to stimulate ATP hydrolysis by the DnaK variants. ATP hydrolysis by all three mutant DnaK proteins was stimulated by peptide substrate and DnaJ to rates very similar to those of wild type DnaK (Table I). Thus, although the elevated $k_{cat}$ values of DnaK756 and DnaK-G455D,G468D indicate altered interaction between the ATPase and substrate-binding domains in these mutant proteins, the interdomain communication and the interaction with DnaJ remained largely intact.

To test the ability of GrpE to stimulate ADP release, steady state ATPase reactions in presence of stoichiometric amounts of DnaJ were performed (Fig. 1). Under these conditions, ATP hydrolysis by DnaK is strongly stimulated, and ADP release becomes the rate-limiting step of the ATPase cycle (11). Addition of increasing amounts of GrpE to wild type DnaK resulted in dramatic stimulation of the overall ATPase activity to a $V_{max}$ of 16 min⁻¹ (420-fold overall stimulation), consistent with earlier findings (11). The effect of GrpE on the DnaJ-stimulated ATPase activity of the DnaK756 mutant protein was reduced in terms of both maximal stimulation (6.2 min⁻¹ at highest GrpE concentration tested) and apparent affinity (saturation not reached at a molar ratio of DnaK:GrpE of 1:3). This result is consistent with the previously observed reduced affinity of GrpE for DnaK756 in the absence of nucleotide (29, 40). In our earlier investigation we demonstrated that the G32D mutation is exclusively responsible for the reduced affinity of GrpE (29). This was confirmed by testing the GrpE-stimulated ATPase activities of the DnaK-G32D and DnaK-G455D,G468D variants (Fig. 1). The DnaK-G32D mutant protein exhibited lower $V_{max}$ values almost indistinguishable from DnaK756. The DnaK-G455D,G468D mutant protein showed a surprisingly high $V_{max}$ of almost 30 min⁻¹ at the highest GrpE concentration tested. Based on further analysis of this mutant protein (see below), we consider this increased $V_{max}$ compared with wild type to result from a stronger stimulation of its ATP hydrolysis by DnaJ.

In summary, the ATPase activity of DnaK756 is affected twofold. First, because of the two carboxyl-terminal mutations, the intrinsic $k_{cat}$ is increased 10-fold. Second, because of the amino-terminal G32D substitution, the GrpE-dependent nucleotide exchange of ADP, produced by the DnaJ-stimulated ATPase activity, is reduced with respect to $V_{max}$ and the apparent affinity of GrpE.

Substrate Interactions

DnaK756 has been shown by size exclusion chromatography-based methods to exhibit reduced binding to carboxymethylated BPTI (42) and AP protein (41). To more precisely characterize these defects in substrate interaction, we determined the dissociation constants ($K_d$) and dissociation rate constants ($k_{off}$) of DnaK756 and the DnaK-G32D and DnaK-G455D,G468D variants for IAANS-labeled peptide ε⁶⁵⁻Q3596-Q444-C (44). Binding of the labeled peptide to DnaK is accompanied by a pronounced increase in fluorescence signal.

Equilibrium titration of DnaK to constant amounts of peptide resulted in similar $K_d$ values of 80 ± 4 nM for the wild type

![Graph](image-url)
and dissociation of tide present to prevent rebinding of labeled peptide. The separation absence of ATP by rapid gel filtration, with excess unlabeled pep-
complexes were separated from free peptide in the presence and
physiological substrate,
firmed in a size exclusion chromatography study with the phys-
DnaK756 on the dissociation of bound substrate was con-
10-fold increased dissociation rate constant, and a 4-fold ele-
the DnaK756 triple mutant protein displays a complex pattern
extent of ATP-dependent peptide release as wild type DnaK,
mentally determined values for
DnaK-G32D was very similar to wild type. From the experi-
apparently reduces the
overall affinity. The G32D substitution, on the other hand,
substitution. We then determined the
k_{off} for DnaK wild type protein was 9.14 ± 0.17 × 10^{-4} s^{-1}
(Table I). DnaK756 exhibited a 9.5-fold higher
and thus a more pronounced alteration in the kinetics of substrate disso-
ciation than in equilibrium binding. An identically increased
k_{off} was found with DnaK-G455D,G468D, whereas the
k_{off} of DnaK-G32D was very similar to wild type. From the experi-
mentally determined values for
K_{d} and k_{off} we calculated the apparent association rate constants (k_{on}) for wild type and
mutant DnaK proteins. Because peptide binding to DnaK in
the absence of ATP has been shown to be a two-step process (9),
this represents a simplification of the real binding process.
The calculated values can nevertheless serve to differentiate
between the effects of the amino- and carboxyl-terminal amino
acid substitutions in DnaK756. Whereas the calculated
k_{on} for DnaK-G455D,G468D is 10-fold higher than for wild type DnaK,
the G32D substitution causes a 2–2.5-fold decrease in the
k_{on}. Both effects seem to be additive, because the DnaK756 triple
mutant protein possesses an only 4-fold elevated
k_{on}.

In summary, the amino acid substitutions of the DnaK756 mutant protein affect substrate binding in two distinct ways. The G455D,G468D substitutions cause a 10-fold increase of both
k_{off} and (calculated) k_{on} resulting in a wild type-like overall affinity. The G32D substitution, on the other hand, apparently reduces the
k_{on} about 2.5-fold without affecting the
k_{off} resulting in a 2.5-fold decrease in affinity. Consequently,
the DnaK756 triple mutant protein displays a complex pattern of substrate binding defects with a 2.5-fold reduced affinity, a
10-fold increased dissociation rate constant, and a 4-fold ele-
vated apparent association rate constant.

The effect of the carboxyl-terminal amino acid substitutions of DnaK756 on the dissociation of bound substrate was con-
firmed in a size exclusion chromatography study with the phys-
ological substrate, σ^{Q2}_{32}-Q132-Q144-C with wild type and
mutant DnaK proteins (5 μM) were loaded onto a G50 nick column
(Amersham Pharmacia Biotech) at 4 °C to separate bound and free
peptide. Immediately prior to separation, unlabeled chase peptide
(black columns) or ATP and unlabeled chase peptide (gray columns)
were added. Release of DnaK-bound peptide was determined as de-
scribed (11, 45). The amount of bound peptide is normalized to
the amount determined in the DnaK-peptide peak in the absence of ATP.

whereas the DnaK756 and DnaK-G455D,G468D mutant proteins
released an only slightly smaller fraction of bound peptide (65 and
50%, respectively). These data demonstrate wild type-like interdo-
main coupling for the DnaK-G32D mutant protein and a significant
coupling potential for DnaK756 and DnaK-G455D,G468D. To-
gether with the wild type-like stimulation of ATP hydrolysis by
σ^{Q2}_{32}-Q132-Q144-C peptide (Table I), severe defects in domain cross-
talk can be excluded for all three mutant proteins.

**Stability of DnaK Tertiary Structure**

The nonconservative amino acid exchanges in DnaK756 have
been shown by partial proteolysis and Fourier transform infra-
red spectroscopy to cause at least local perturbations of the
DnaK tertiary structure (42, 43). To analyze the contributions of the amino- and carboxyl-terminal mutations to the destabi-
lization of DnaK756, we performed partial proteolysis experi-
ments using the broad specificity protease papain.

Papain digestion of wild type DnaK generated two stable
fragments of 44 and 27 kDa apparent molecular mass, comprising
the ATPase and substrate-binding domains, respectively (Fig. 3 and Ref. 53). These fragments result from cleavage
within the linker region between residues 386–393 (53). In contrast, in case of the DnaK756 mutant protein, the 27-kDa
substrate-binding domain fragment did not accumulate but
was further degraded to products of less than 20 kDa, indicat-
ing destabilization of the substrate-binding domain. In addi-
tion, the 44-kDa fragment corresponding to the ATPase domain
was also slightly destabilized. Papain digestion of DnaK-
G455D,G468D demonstrated that its two mutational alter-
ations result in destabilization of the substrate binding but not
the ATPase domain (Fig. 3). The proteolysis pattern of DnaK-
G32D showed that the single glycine-to-aspartate substitution destabilizes the 44 kDa ATPase fragment to a similar extent as in
DnaK756. Surprisingly, the G32D substitution seemed to
slightly destabilize the 27-kDa substrate-binding domain frag-
ment as well, although less dramatically than the G455D,
G468D double mutation does. After 120 min of incubation,
the 27-kDa fragment of DnaK-G32D can be estimated to be 5–10-
fold less populated as compared with wild type DnaK but about
5–10-fold more populated in comparison to DnaK756. (The
abundance of the 44-kDa fragments of DnaK756 and DnaK-
G32D can be estimated to be about 10% of those of wild type
DnaK and DnaK-G455D,G468D.) This finding might indicate
that destabilization of the ATPase domain in DnaK-G32D in-

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**Domain Coupling**

We investigated the ability of the mutant DnaK proteins to
couple binding and hydrolysis of ATP with substrate binding by
testing whether they release radioactively labeled σ^{Q2}_{32}-Q132-
Q144-C peptide upon addition of ATP. Preformed DnaKpeptide
complexes were separated from free peptide in the presence and
absence of ATP by rapid gel filtration, with excess unlabeled pep-
tide present to prevent rebinding of labeled peptide. The separation
was performed at 4 °C, where ATP hydrolysis is greatly reduced
and dissociation of σ^{Q2}_{32}-Q132-Q144-C peptide in the absence of ATP
is negligible on the time scale of the separation (approximately 5
min). About 80% of bound peptide was released from wild type
DnaK upon addition of ATP (Fig 2). DnaK-G32D showed the same
extent of ATP-dependent peptide release as wild type DnaK,
induces a subtle conformational perturbation of the substrate-binding domain by interdomain cross-talk and might help to explain the lower (calculated) $k_{\text{cat}}$ for peptides of this mutant protein (Table I).

We confirmed these findings by partial proteolysis experiments using trypsin (data not shown). Partial trypsic degradation allows monitoring of overall structural integrity as well as nucleotide-dependent domain coupling of DnaK (55). We found that (i) DnaK756 and DnaK-G455D,G468D were degraded significantly faster than the wild type protein; (ii) DnaK-G32D displayed degradation products and kinetics almost identical to the wild type; and (iii) ATP-dependent structural changes indicative of domain coupling were wild type-like for DnaK-G32D, less significant but detectable for DnaK756, and not detectable for DnaK-G455D,G468D.

Taken together, partial proteolysis experiments with two different proteases consistently show that the two carboxy-terminal amino acid substitutions in DnaK756 and DnaK-G455D,G468D destabilize the substrate-binding domain. In addition, papain cleavage indicates a minor destabilization of the ATPase domain by the G32D substitution. Trypsin cleavage demonstrates that DnaK756 and DnaK-G32D exhibit ATP-induced conformational changes.

**Chaperone Function in Vivo**

To characterize the consequences of the mutational alterations in DnaK756 and derivatives for DnaK chaperone function, we performed functional tests in vivo and in vitro. We analyzed in vivo the ability of the plasmid-encoded alleles $\text{dnaK-G32D}$ and $\text{dnaK-G455D,G468D}$ (nomenclature analogous to the proteins), along with $\text{dnaK756}$ and $\text{dnaK}^+$. To complement the temperature-sensitive growth and bacteriophage $\lambda$ resistance of $\Delta\text{dnaK52}$ mutants lacking DnaK (56, 57). Expression of the plasmid-encoded $\text{dnaK}$ alleles was controlled by a T7 promoter/lacO$_{101}$ promoter/operator system, allowing tight repression in the presence of IPTG and regulated expression upon induction with isopropyl-$\beta$-D-galactopyranoside (45).

Expression of plasmid-encoded $\text{dnaK}^+$ and $\text{dnaK-G455D}$, $\text{G468D}$ allowed growth at $42^\circ$C after induction with $50-500\mu$m isopropyl-$\beta$-D-galactopyranoside (Table I). In contrast, expression of $\text{dnaK756}$ and $\text{dnaK-G32D}$ did not allow growth of the cells at $42^\circ$C. With respect to $\lambda$ sensitivity, expression of $\text{dnaK}^+$ fully supported lytic growth of $\lambda^{\text{vir}}$ at $30^\circ$C in $\Delta\text{dnaK52}$ cells, whereas expression of $\text{dnaK756}$ did not support lytic growth, and $\text{dnaK-G32D}$ and $\text{dnaK-G455D,G468D}$ allowed for moderate levels of propagation of $\lambda^{\text{vir}}$ (Table II).

Together, these data demonstrate that both $\text{dnaK-G32D}$ and $\text{dnaK-G455D,G468D}$ possess partial chaperone function in vivo, with $\text{dnaK-G32D}$ exhibiting more pronounced defects than $\text{dnaK-G455D,G468D}$. DnaK756, on the other hand, did not exhibit any chaperone function in vivo, consistent with the original description of its phenotype (58).

**Chaperone Function in Vitro**

**Initiation of $\lambda$ DNA Replication—The in vitro activities of the DnaK756, DnaK-G32D, and DnaK-G455D,G468D mutant proteins were investigated in two well-characterized assays indicative of the chaperone function of DnaK. First, their activities in the disassembly of protein complexes was assayed by the ability to assist the initiation of $\lambda$ DNA replication in vitro (48). The essential role of DnaK in this process is to promote disassembly, in cooperation with the DnaJ co-chaperone, of the nucleoprotein preinitiation complex formed at the origin of replication of the $\lambda$ chromosome, thereby activating DnaB helicase (59–61). GrpE is not strictly required in this in vitro system but strongly increases the apparent specific activity of DnaK (59, 61). Initiation of $\lambda$ DNA replication in the presence and absence of GrpE was strictly DnaK-dependent and reached an optimal level at approximately 2 and 5 $\mu$g of added DnaK, respectively (Fig. 4, a and b). In contrast, the DnaK756 mutant protein showed no (Fig. 4b) or strongly reduced (Fig. 4a) activity in the absence and presence of GrpE, respectively. The DnaK-G32D and DnaK-G455D,G468D mutant proteins displayed specific activities in the presence of GrpE that were very similar to or slightly lower than the wild type protein with optima at approximately 2 and 5 $\mu$g of mutant protein, respectively (Fig. 4a). Surprisingly, DnaK-G455D,G468D was completely inactive in the absence of GrpE, whereas DnaK-G32D showed a similar, reduced activity as in the presence of GrpE (Fig. 4b). To elucidate this remarkable effect of GrpE on DnaK-G455D,G468D, we titrated GrpE into replication reactions with constant, approximately half-optimal amounts of wild type and mutant DnaK proteins (Fig. 4c). Consistent with the results shown in Fig. 4b, wild type DnaK and DnaK-G32D showed significant activity even in absence of GrpE and responded to increasing GrpE concentrations in a similar manner, with an optimum at 75–100 ng of GrpE. DnaK-G455D,G468D displayed no activity in absence of GrpE but showed a strong increase in activity between 10 and 75 ng of GrpE present. The DnaK756 protein, which was present in this experiment at 6.4-fold higher concentration than the three other DnaK proteins, interestingly showed significant activity at in-

### Table II

| Plasmid-encoded dnaK allele | Growth | Plaque formation |
|-----------------------------|--------|-----------------|
| 30°C | 42°C | 0–500 | 0 | 500 | 250 | 500 | 250 |
| None | + | + | + | + | + | + | + | + |
| dnaK$^+$ | + | + | + | + | + | + | + | + |
| dnaK756 | + | + | + | + | + | + | + | + |
| dnaK-G32D | + | + | + | + | + | + | + | + |
| dnaK-G455D,G468D | + | + | + | + | + | + | + | + |

* Ability to form colonies as compared with the dnaK$^+$ control at 30°C; +, normal number and size of colonies; (+), reduced number and size of colonies; −, no colonies.

* Number of plaque forming units normalized to the dnaK$^+$ control. ND, not determined. * IPTG concentration (mM).

* IFP concentration (mM).
termediate and high GrpE concentrations, suggesting that the inability of the dnaK756 allele to support lytic growth of \(\lambda\) bacteriophage \textit{in vivo} might result from reduced affinity of DnaK756 for GrpE.

In summary, the ability of all three DnaK mutant proteins to assist in the initiation of \(\lambda\) replication is affected by GrpE in a complex way. DnaK756 is largely inactive but gains activity at high DnaK and GrpE concentrations. DnaK-G32D displays a reduced specific activity but an otherwise wild type-like dependence on GrpE concentration. DnaK-G455,468D is completely inactive in the absence but almost as active as the wild type in the presence of GrpE.

\textbf{Luciferase Refolding}—The activity of the DnaK proteins in protein refolding was assayed by their ability to assist refolding of denatured firefly luciferase. Denaturation by guanidine hydrochloride presumably brings about complete unfolding of luciferase. Upon dilution from denaturant, refolding proceeds \textit{via} two aggregation-prone folding intermediates (62). Efficient refolding of luciferase requires DnaK, DnaJ, and GrpE at defined molar ratios (51, 63). Wild type DnaK assisted reactivation of guanidine hydrochloride-denatured luciferase to more than 80% of the activity present prior to denaturation (Fig. 5). In contrast, in the presence of DnaK756 mutant protein the refolding yield was only slightly higher than without DnaK, less than 20% (Fig. 5). Increasing the DnaK756 concentration did not result in higher yields (data not shown). Consistent with the results of the \textit{in vitro} \(\lambda\) replication experiments in the presence of GrpE, the DnaK-G455D,G468D variant assisted luciferase refolding to yields similar to those of wild type DnaK.

We tried to reproduce the remarkable effects produced by varying GrpE concentrations in the \textit{in vitro} \(\lambda\) replication assay by performing luciferase refolding assays with guanidine-denatured luciferase in the presence of two different fixed DnaK concentrations (0.8 and 1.2 \(\mu\)M) and varying concentrations of GrpE of up to 6 \(\mu\)M (data not shown). Wild type DnaK consistently showed an optimum at molar ratios of DnaK and GrpE of 1:0.5 to 1:1 in agreement with previous reports (Ref. 24 and data not shown). The DnaK-GG455,468D variant responded to variations in GrpE concentration in a very similar way. On the other hand, even very high GrpE concentrations did not result in significant increase in chaperone activity with the DnaK756 and DnaK-G32D mutant proteins (data not shown).

\textbf{Luciferase Aggregation}—The results obtained \textit{in vitro} with DnaK-G32D were surprising in light of the significant activity of this variant in \(\lambda\) replication but lack of activity in luciferase refolding. We therefore investigated the defect of this mutant protein in assisting luciferase refolding in more detail by determining in light scattering experiments its potential to prevent luciferase aggregation (51, 52). Efficient prevention of luciferase aggregation has been shown to require the presence of DnaJ, DnaK, and MgATP (51), acting in a highly coordinated coupling mechanism (5, 18). DnaK-G32D suppressed luciferase aggregation with a similar efficiency as wild type DnaK (Fig. 6A), indicating that the DnaJ-dependent mechanism to lock-in substrates is not strongly affected by the G32D substitution. To analyze GrpE-dependent complex dissociation, we added GrpE to preformed DnaK-(DnaJ)-luciferase complexes in the presence of ATP and monitored in parallel aggregation and reactivation of luciferase. With both wild type and DnaK-G32D proteins, addition of GrpE did not induce major luciferase aggregation as compared with the absence of the chaperones (Fig. 6B). However, whereas with wild type DnaK efficient reactivation of luciferase was observed upon addition of GrpE, no reactivation occurred with DnaK-G32D (Fig. 6C), even when 10-fold higher concentrations of GrpE were added (data not shown). Therefore, it can be speculated that luciferase dissociates from DnaK-G32D in a conformation that is neither prone to aggregation nor committed to refold to the native state.

\textbf{DISCUSSION}

We report a comprehensive analysis of defects of the DnaK756 mutant protein caused by the amino-terminal G32D and the carboxyl-terminal G455D and G468D substitutions. This dissection provides a mechanistic understanding for the numerous phenotypes reported for this classical and widely used mutant protein. We found that DnaK756 has (i) 10-fold elevated basal ATPase activity that is stimulated efficiently by DnaJ and substrate peptide but less efficiently compared with wild type DnaK by GrpE; (ii) 2.5-fold decreased affinity (\(K_a\)) for substrate peptides, resulting from a 10-fold increased \(k_{on}\) and a 4-fold decreased apparent \(k_{on}\); (iii) at least partial ability to
release peptide substrate upon addition of ATP; and (iv) conformational destabilization of the substrate-binding domain and, to a lesser extent, of the ATPase domain.

Most of these defects can be attributed to either the G32D or the G455D and G468D amino acid substitutions in a straightforward manner. The G32D exchange is solely responsible for the reduced affinity to GrpE in the presence (Fig. 1) and absence (29) of ATP, as well as for a slight destabilization of the ATPase domain in the absence of ATP (Fig. 3). The G455D, G468D double substitution, on the other hand, causes the elevated basal ATPase activity (Table I), the destabilization of the substrate-binding domain (Fig. 3), the increased $k_{off}$ for substrate (Table I), and potentially a moderate defect in interdomain communication (see below).

Less clear is the influence of the G32D substitution on substrate binding. Because the $k_{off}$ for substrate is identical for DnaK-G32D and wild type DnaK, the reduced affinity has to be attributed to slower association kinetics. Peptide binding to DnaK in the absence of ATP has been reported to be a two-step reaction (9). The nature of the two steps is unknown, and at least two possible scenarios are plausible: (i) The two steps represent an initial encounter of DnaK and substrate, followed by a slower isomerization step (9). In this case, the slower substrate binding by DnaK-G32D would probably result from an altered initial encounter, because the $k_{off}$ as well as the stability of the substrate-binding domain are unaffected in this mutant protein. It is an intriguing possibility that the exposed loop, in which Gly$^{32}$ has a crucial structural role (29), is physically contacting the carboxyl-terminal domain(s) such that substrate binding is influenced. (ii) DnaK is known to undergo reversible oligomerization involving its substrate-binding site (47). The faster first step could therefore represent binding of substrate to the readily accessible monomeric subpopulation of DnaK, whereas the slow second phase could correspond to binding to the oligomeric subpopulation that is only accessible to substrate after a rate-limiting monomerization step. In this scenario, DnaK-G32D might simply exhibit a slightly shifted monomer/oligomer equilibrium.

The nonconservative glycine-to-aspartate amino acid exchanges in DnaK756 lead to a destabilization of the substrate-binding domain and, to a much lesser extent, the ATPase domain, as shown by partial proteolysis experiments (Ref. 42 and Fig. 3). These findings are consistent with a Fourier transform infrared spectroscopy study that demonstrated perturbations of secondary and tertiary structural elements in DnaK756, with concomitant (i) higher hydrogen exchange rates and (ii) a decrease of the midpoint of thermal denaturation from 50 to 35 °C (43). It should however be noted that the study by Banecki et al. (43) was performed under conditions favoring inactivation/destabilization of DnaK756, i.e. absence of K$^+$, Mg$^{2+}$, and nucleotide, high protein concentration, storage at room temperature for days.

The destabilizing effects of the amino acid substitutions in DnaK756 can now be interpreted in molecular detail on the basis of the solved structures of the domains involved (Ref. 27 and 30 and Fig. 7). The G32D exchange most likely destabilizes the substrate-binding domain (Fig. 3), the increased $k_{off}$ for substrate (Table I), the destabilization of the ATPase domain (Ref. 42 and Fig. 3) and, to a lesser extent, of the ATPase domain.

A

**FIG. 6.** Defect of DnaK-G32D in luciferase reactivation. A and B, DnaK-G32D prevents aggregation of denatured luciferase. Guanidine hydrochloride-denatured luciferase was diluted into refolding buffer containing no additions (○), DnaJ alone (▲), DnaJ and bovine serum albumin (▼), DnaJ and wild type DnaK (■), or DnaJ and DnaK-G32D (●). Luciferase aggregation was determined by monitoring light scattering. The final concentrations were: 160 nM luciferase, 80 nM DnaJ, 1.6 μM DnaK. In B, GrpE (final concentration, 800 nM) was added to the aggregation reactions after 35 min (arrow). C, addition of GrpE to DnaK-G32D-DnaJ-luciferase complexes does not result in luciferase reactivation. Reactivation of luciferase after addition of GrpE to aggregation reactions (arrow in B) was determined as described (52) and normalized to luciferase activity prior to denaturation. **Dashed lines,** samples taken from aggregation experiment (B) after addition of GrpE; **closed lines,** standard refolding experiment (control). ○, no DnaK; ■, wild type DnaK; ●, DnaK-G32D.
that is capable of accommodating the flexible linker between the ATPase and substrate-binding domains (30). It adopts Ramachandran angles that are slightly forbidden for residues except glycines, raising the possibility that the mutation of residue 455 to aspartate structurally rearranges its neighborhood. In addition, there is a steric conflict with residue Ile499 in $\beta$-strand 8. Mainly involved in the local perturbations are probably residues Leu454, which is the direct neighbor of Gly455, and Ile501, which is adjacent to Ile499. Leu454 and Ile501 form one side of the hydrophobic pocket described above.

The G468D mutation is expected to distort the geometry of loop 5,6 of the substrate-binding domain (30). The neighboring residue Arg467 of this loop forms a salt bridge to residue Asp540 in the lid-forming $\alpha$-helix B and is thus important in stabilizing the closed conformation of the substrate-binding cavity (Fig. 7B, inset). Disruption of the salt bridge has been implicated in the mechanism of substrate dissociation (30), and it has been speculated that ATP binding to the ATPase domain might specifically induce disruption of this salt bridge, thereby providing the structural basis for ATP induced substrate dissociation (8, 30). Arg467 has indeed been shown by partial proteolysis experiments to become the major tryptic cleavage site in the presence of ATP (55). The Ramachandran angles of Gly468 are slightly outside the allowed region for loops, making a distortion of the whole loop by the G468D mutation feasible. The geometry of this loop is possibly stabilized by an intra-loop H-bond between residues 466 and 469 (3.44 Å distance between 466 O and 469 N), which most likely would be destroyed by the mutation G468D. This mutation may also disrupt the adjacent salt bridge Arg467–Asp540 and thus induce the predicted destabilization of the outer part of the substrate-binding domain (30). The observation that partial proteolytic degradation of DnaK756 and DnaK-GG455,468D with trypsin is rapid and relatively insensitive to the nucleotide state (data not shown and Ref. 42) suggests that Arg467 is constitutively accessible to the protease, consistent with a structural perturbation of loop 5,6. Although our approach did not allow us to determine the relative contributions of the G455D and G468D amino acid substitutions to the defects of DnaK-G455D,G468D and DnaK756, it is tempting to speculate that the major effect of the G468D exchange is the structural destabilization of the substrate-binding domain, whereas the G455D exchange might cause slight coupling defects as evident from the elevated basal
Functional Dissection of the DnaK756 Mutant Chaperone

ATPase activities and the slightly reduced potential of ATP-dependent peptide release (Fig. 2).

It has been reported that the DnaK756 mutant protein releases bound substrates upon addition of ATP less efficiently than wild type DnaK (38, 42), and that its tryptic cleavage pattern is ATP-independent (42). These findings had been interpreted to suggest (42) that the coupling between ATP binding and structural alterations in the substate-binding domain leading to substrate release is compromised in DnaK756. However, the experimental basis for this conclusion is insufficient. The extent of ATP-induced peptide release was determined after considerable incubation/ separation periods at room temperature and under conditions that did not prevent rebinding of already released substrate (38, 42). Consequently, the majority of DnaK756 in these experiments was present in the high affinity ADP state because of its elevated ATPase activity, whereas a much smaller subpopulation of wild type DnaK was present in the ADP state because of its lower ATPase activity. Consistent with this interpretation, we were able to demonstrate significant ATP-dependent substrate release by DnaK756 under conditions that prevent rebinding of dissociated peptide and minimize ATP hydrolysis, i.e. by adding excess ATP and excess competitor substrate immediately before separation at 4 °C (Fig. 2). The failure to detect wild type-like ATP-dependent conformational changes in partial proteolysis experiments (42) might similarly be the consequence of the accumulation of the ADP state of DnaK756 during the incubation period. A more principal limitation of this approach might be the presumably constitutive exposure of the Arg 

3467 tryptic cleavage site because of the G468D exchange in DnaK756 (see above), rendering the most significant indicator for ATP-induced conformational changes in the wild type DnaK protein, i.e. a 21-kDa fragment (55), independent of the nucleotide state and thus useless in DnaK756. Positive evidence in favor of intact domain communication comes from the ATPase measurements. The stimulation of the basal ATPase activity by peptide or DnaJ, reflecting transmission of binding signals from the substrate-binding site to the ATPase domain, is unaffected in DnaK-G32D and at least largely intact in DnaK756 and DnaK-G455D,G468D (Table I). For the latter two mutant proteins, the stimulation factors are lower compared with the wild type protein because of their increased basal activities, whereas the absolute stimulated rates are very similar to wild type DnaK protein. Finally, the most sensitive indicator for domain coupling is chaperone activity itself. Although we were not able to demonstrate the same extent of domain coupling for DnaK-G455D,G468D as for wild type protein by direct measurements, the significant chaperone activity of this variant in different assay systems in vivo and in vitro provides convincing proof of the retention of intact domain communication.

The G32D and G455D,G468D substitutions affect DnaK chaperone function in different ways and to different extents. Although the DnaK-G455,G468D mutant protein possesses a significantly destabilized substrate-binding domain resulting in higher susceptibility to proteolytic degradation, faster substrate dissociation and elevated basal ATPase activity, its chaperone function is not compromised dramatically even at elevated temperatures of 42 °C (Table II and data not shown). It can therefore be assumed that interactions with substrates and/or DnaJ stabilize the mutant substrate-binding domain at least to some extent. In contrast, DnaK-G32D possesses major defects in chaperone functions in vivo and in vitro despite the lack of defects in most of the biochemical and domain coupling activities tested. The only exception is the inefficient stimulation of ATPase activity by GrpE (Fig. 1), consistent with the weakened physical interaction of DnaK-G32D and GrpE in the absence of ATP (29). The stronger phenotypes caused by the amino-terminal G32D exchange have been revealed previously in two independent investigations, performed prior to sequencing of the dnaK756 allele. Cegielska and Georgopoulos (39) concluded that the dnaK756 mutation is located within the amino-terminal 30% of the dnaK gene. Ezaki et al. (64) mapped and sequenced the seg-1 allele conferring temperature sensitivity in F plasmid propagation and found that it is a mutant dnaK allele differing from wild type solely by a G32D exchange. Extending these findings, our data show additivity of the defects caused by the G32D and G455D,G468D exchanges in vivo, at least in λ replication. The G32D exchange alone does not confer a clear λ phenotype (Table II) to dnaK, so that additional minor deficiencies because of the G455D,G468D exchanges were probably required to pick up the dnaK756 allele in the genetic screen for a λ phenotype (1). In contrast, upon selection for temperature sensitivity, the defects of DnaK-G32D alone are severe enough to identify seg1-1/dnaK-G32D (Ref. 64 and Table II).

In this context, it is interesting to note that even the DnaK756 triple mutant protein possesses some chaperone activity in the λ in vivo replication system (Fig. 4, a and c). This is consistent with similar data by Zylicz et al. (61), who determined replication activity up to a maximal amount of 5 μg of DnaK756, compared with 20 μg in Fig. 4a. At both high DnaK756 and GrpE concentrations, up to 20% of the maximal activity of DNAK wild type protein was detected (Fig. 4c). This severely compromised but detectable residual activity of the DnaK756 mutant protein in vitro indicates that it is probably at the borderline of detectable chaperone function in vivo and in vitro, rather than being a knock out mutant protein. This conclusion is confirmed by the activity of the DnaK756 mutant protein in assisting λ DNA replication from initiation complexes containing a mutant AP protein, λ at A66 (65). This mutant AP protein binds DnaB helicase less tightly, so that the reduced affinity of DnaK756 for substrates is still sufficient to liberate DnaB from λ.

A residual chaperone activity of DnaK756 could be observed in vitro in λ replication but not in luciferase refolding. Compared with λ replication, luciferase refolding represents a much more stringent system with respect to chaperone requirements. Liberation of DnaB from the λ replication preinitiation complex requires only one or few rounds of DnaK binding to AP (48, 59, 61), whereas assistance in luciferase refolding requires multiple rounds of DnaK binding and release in a kinetic competition with irreversible aggregation processes (52). This higher stringency is even more obvious for the DnaK-G32D mutant protein, which has pronounced activity in λ replication in vivo and in vitro but is completely inactive in luciferase refolding. Our data show that the ability to prevent aggregation of non-native luciferase by rapid, DnaJ-mediated binding is almost unaffected in DnaK-G32D (Fig. 6A). In contrast, nucleotide exchange and/or luciferase release are severely affected, because addition of GrpE does not lead to productive luciferase refolding. This is not simply an effect of reduced affinity for GrpE (Ref. 29 and Fig. 1), because at 10-fold higher concentration of GrpE the same defect was observed. Rather, the kinetics of interaction between GrpE and DnaK-G32D might be affected more severely than the overall apparent affinity. Considering that the chaperone cycle of DnaK in presence of substrate, ATP, DnaJ, and GrpE is in a delicate kinetic balance (18, 24), then even slightly altered kinetics of interaction between GrpE and DnaK-G32D could provide an explanation for the apparent
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