INTERACTION BETWEEN THE *ESCHERICHIA COLI* HEAT SHOCK PROTEINS DnaK AND GrpE

by

Anna Louise Maddock

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of a dissertation submitted by

Anna Louise Maddock

This dissertation has been read by each member of the following supervisory committee and by majority vote has been found to be satisfactory.

Chair:

Tom Alber

15 Nov 93

Dana

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Ann W. Hart
Dean of The Graduate School

ABSTRACT

The DnaK, DnaJ and GrpE *Escherichia coli* proteins have been shown to work together as a chaperone system which functions to help dissolve protein aggregates, such as heat inactivated RNA polymerase, and to aid in assembly and disassembly of protein structures, such as that found at the origin of λ DNA replication. The DnaK protein is thought to be divided into two distinct domains, the amino-terminal ATPase domain, and the carboxyl-terminal substrate interaction domain. In the chaperone system, DnaK provides the major chaperoning activities, whereas DnaJ and GrpE act as DnaK's cohorts, helping DnaK to better interact with its substrates, and to be recycled.

In order to better understand the interaction between the DnaK and GrpE proteins, single copy and multicopy mutations in *dnaK* that could function to allow *E. coli* growth in the absence of *grpE* were selected and characterized both genetically and biochemically. The single copy mutations, *dnaK*325 and *dnaK*332 are point mutations in the amino-terminal, ATPase domain of DnaK. The multicopy mutations result in truncations, from 94 to 248 amino acids from the carboxyl-terminal end of the DnaK protein.

Genetic and biochemical characterization of these mutants indicate that the GrpE protein helps DnaK to interact with and to release its substrates through (a) maintaining a proper balance between the ATP- and ADP-bound forms of DnaK, and (b) influencing the conformation of DnaK. They further indicate that the carboxyl-terminus of the DnaK protein acts as a negative regulator of the amino-terminal ATPase activity, and that the amino-terminus must be involved in substrate interaction.

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CHAPTER 1

INTRODUCTION TO THE *ESCHERICHIA COLI* CHAPERONE SYSTEMS

At a given moment in the bacterial organism *Escherichia coli*, there are many proteins being translated, folded, transported, secreted, complexed with other proteins, unfolded or degraded in order that the organism might carry out the functions it needs to survive and propagate itself. The pioneering work of Anfinsen suggested that the task of protein folding was accomplished by the proteins themselves, i.e., that a protein's three-dimensional structure is encoded in its amino acid sequences in much the same way that its amino acid sequence is encoded in the DNA sequence of its gene. In this way, as a nascent peptide emerges from the ribosome, it would begin to assume its three-dimensional structure as dictated by its amino acid sequence. Should the protein be unfolded at some point, it could simply refold itself, again, according to the dictates of its amino acid sequence (1).

Although this model is certainly correct for many proteins under various circumstances, it does not account for certain events that may occur upon folding of a particular protein. For instance, as enumerated above, the protein may require secretion or translocation, events that would require it to be either maintained in an unfolded state or actively unfolded and then refolded once it had reached its final destination. Alternatively, the protein may need to form a complex with other proteins in order for

it to function properly or to form a multimeric structure. In this case, it may be that the protein is capable of forming complexes with several proteins that are present in the cell at once, but that forming a complex with only one or a subset of these proteins is appropriate. Thus, there must be a mechanism within the cell that allows the formation of only appropriate protein complexes at a given time. The protein may form aggregates with like proteins as it begins to fold upon emerging from the ribosome, and thus may simply require assistance in folding as it is translated. An organism may encounter stresses such as a sudden shift in temperature or pH, or exposure to a toxic substance, events that may cause a folded protein to unfold, misfold or to form inappropriate aggregates with other proteins. Recovery of the organism from such events would require that the inappropriate aggregates be dissolved, the unfolded or misfolded proteins be refolded properly or degraded, and proper complexes reformed -- trials not accounted for by the original protein folding model.

Over the last several years, it has become clear that many of the above tasks, both under normal and stress conditions, are carried out by a group of proteins collectively referred to as molecular chaperones, many of which were originally discovered as heat shock proteins. It has been found that in response to unfavorable

conditions, living organisms will cease normal protein synthesis and commence a transient, yet vigorous production of a small set of proteins. These proteins are commonly referred to as "heat shock proteins" or "stress proteins," and over the last several years, much evidence has indicated that a subset of the stress proteins exists primarily to help other proteins to fold, unfold, form complexes, be disaggregated or be degraded as a particular situation calls for (reviewed in 15, 41, 59, 17, 19, 16). These particular stress proteins are conserved in nature and are termed "chaperones" or "chaperonins" because of their ability to assist in appropriate complex formation. Such proteins are produced at high rates under stress, and at substantially reduced rates under normal conditions. The chaperones which are also heat shock proteins include the GroES (Hsp10) and GroEL (Hsp60) proteins, the Clp (Hsp104) family of proteins and the DnaK (Hsp70), DnaJ (Hsp40) and GrpE proteins of *E. coli* and their prokaryotic and eukaryotic homologs. Though none of these families of proteins were originally discovered as chaperones, in recent years much evidence has accumulated which demonstrates such activities in these proteins and indicates that many of the chaperones work in concert with each other as a system to accomplish their given tasks (for reviews see 22, 59, 15, 14).

In this chapter, I wish to concentrate on the discoveries and properties of the *E. coli* chaperone systems, and focus on the accumulated evidence which points to the mode of action of one of these systems: the DnaK-DnaJ-GrpE system.

Discovery of the Heat Shock Proteins

In the early 1960s, it was observed that when Drosophila larvae were exposed to stresses such as a sudden increase in temperature, release from anoxia, addition of 2,4-dinitrophenol or addition of sodium azide, seven new puffs appeared on the giant chromosomes of their salivary glands, while other puffs present before addition of the stressor receded or disappeared (51, 52, 53). When the exposure to the stressor was transient (lasting only 30 min), the puffs persisted for approximately one hour. If it were prolonged, they would persist longer, but would eventually disappear. It was later found that the chromosome puffs were the result of the intense transcription of a small number of genes, and that while these gene products were being synthesized, the production of other proteins was inhibited (63).

Eventually, it was found that this heat shock or stress response is a universal one and can be induced in other organisms, from chickens (26) to plants (5) to yeast (38) to *E. coli* (29), and in response to many other

stressors as well (reviewed in 41). Although the response varies from organism to organism in the quantity of proteins produced, it is consistent in quality as defined by a rapid and transient synthesis of a small set of proteins accompanied by the inhibition of synthesis of the proteins produced prior to the heat shock (reviewed in 41).

The *E. coli* Heat Shock Proteins

In *E. coli*, there are approximately 20 heat shock or stress proteins whose expression is transiently increased in response to heat shock and other stressors. These proteins are readily seen upon shifting cells which are growing at 30°C to 42°C, pulse labeling them, and examining their extracts by 2-dimensional polyacrylamide gel electrophoresis (43). However, identification of the corresponding genes for these proteins is a continuing process. As with all heat shock responses the increase in expression of these genes is transient, increasing rapidly immediately following the shift in temperature, but falling off after approximately 8 min, and returning to previous levels after 15 min (43).

Each of the known *E. coli* heat shock genes is transcriptionally regulated by the product of the *rpoH* gene, the σ^{32} transcription factor (20, 21). The promoter regions of many of these genes have been sequenced and found to contain, in addition to any other housekeeping

promoters, the sequences CGGCAA and CTGAA at -10 and -35 sites, which is the consensus for σ^{32} transcriptional regulation (12). Furthermore, the expression of the *E. coli* heat shock genes has been shown to correlate well with the intracellular levels of σ^{32} (reviewed in 15, 19).

The identified heat shock genes of *E. coli* include *groES groEL*, *dnaK*, *dnaJ*, *grpE*, *clpB*, *clpP*, *clpX*, *lon*, *htpG*, *lysU*, *rpoD*, *htrC*, *htrM*, and *htpY*. The *groES*, *groEL*, *dnaK*, *dnaJ* and *grpE* gene products were originally discovered as *E. coli* factors required for the growth of bacteriophage λ (reviewed in 14, 15, 16). Each was later found to be essential to *E. coli* growth and to be highly conserved in nature (reviewed in 14, 15, 16).

The *groES* and *groEL* genes form an operon at 94.2 min on the *E. coli* chromosome (61, 62). The *groEL* and *groES* gene products are thought to function together as a chaperone system to assist proteins in folding properly by preventing their aggregation. In this system, GroEL is thought to carry out most of the chaperone functions, with GroES acting as GroEL's assistant, regulating GroEL's ATPase activity and conformation, and possibly helping GroEL to release substrates (reviewed in 22).

Like the GroEL and GroES proteins, the DnaK, DnaJ and GrpE proteins are thought to function together as a chaperone system team. As with the GroE system, in this system, DnaK carries out most of the chaperoning

activities, and DnaJ and GrpE act as DnaK's assistants (for reviews, see 22, 15, 16; and see below for further details).

The ClpB, ClpX and ClpP proteins are components of an emerging, complex protease system in *E. coli* (18, 67; reviewed in 59). The ClpP protein has been shown to possess a weak peptidase activity, incapable of cleaving full-length proteins by itself, but to be strongly induced by other Clp family members, such as ClpA and ClpX, to cleave specific target proteins (23, 24, 67). It has been proposed that the ClpA and ClpX proteins may act as molecular chaperones, sequestering substrates and presenting them to ClpP for degradation (67, and reviewed in 59). ClpB is thought to have dual functions as a proteolytic regulator and molecular chaperone acting to protect certain cellular components from heat stress (46).

The Lon-protease is an ATP dependent serine protease, thought to be involved in the degradation of abnormally folded proteins (10, 11, 60). Lon-dependent cleavage of abnormal proteins *in vivo* is partially dependent on the DnaK and DnaJ proteins. It may be that *in vivo*, DnaK and DnaJ are responsible for binding to such proteins and presenting them to Lon for degradation (25, 56, 60).

The *htpG* gene product is the Hsp90 heat shock protein homologue of *E. coli* (3). This gene can be deleted from *E. coli* with no apparent adverse effects, except at very high

temperature (4). Although no specific function has yet been ascribed to Hsp90, it has been found to have an ATPase activity and to be able to bind to the σ^{32} protein and to various members of the peptidyl prolyl isomerase family of proteins *in vitro* (42). Such results indicate that the HtpG protein may have a redundant function in *E. coli* as a molecular chaperone, especially at very high temperatures, or as a factor involved in regulation of the heat shock response.

The *lysU* gene encodes one of two *E. coli* lysyl-tRNA synthases. This gene product is expressed uniquely under stress conditions. It is thought that one important function of the *lysU* gene, aside from charging $tRNA^{lys}$ with lysine, is that of synthesizing the dinucleotide AppppA, which is thought to be a signal of stress (6, 28).

The *rpoD* gene encodes the σ^{70} subunit of RNA polymerase (RNAP). This factor is responsible for allowing RNAP to recognize the promoters of housekeeping genes expressed under normal, nonstress conditions (43). It is thought that this product may be under control of the stress response as a means of negatively regulating the response. One possibility is that as the levels of σ^{70} rise in the cell, it is able to compete with σ^{32} for binding to RNAP. Once the level of σ^{70} is sufficiently high, it replaces σ^{32}, and RNAP can re-initiate the efficient transcription of housekeeping genes.

htrC, *htrM/rfaD*, and *htpY* were discovered as genes whose products are required for the growth of *E. coli* at high temperature. The *htrC* gene encodes a basic polypeptide of approximately 21,000 KDa. Its sequence shares limited homology with that of the *spoIIA* gene which encodes a *B. subtilis* transcription factor. Although no specific function has yet been ascribed to the *htrC* gene product, inactivation of the *htrC* gene results in overproduction of heat shock proteins at all temperatures, a general defect in cellular proteolysis, cell filamentation at intermediate temperatures, and cell lysis and death at temperatures above 42°C. Such characteristics have led to speculation that *htrC* may encode a negative transcriptional regulator of the heat shock response (49).

htrM/rfaD encodes an enzyme required for the synthesis of lipopolysacharide (LPS) (50, 47). Why such an enzyme is partially under the control of the heat shock response and its role in response to stressful situations is not as yet understood.

The *htpY* gene resides 700 base pairs upstream of the *dnaK dnaJ* operon. It encodes a 21,000 KDa product. Mutations in this gene lead to a generalized decrease in the *E. coli* heat shock response, indicating that the HtpY protein may play a positive role in the regulation of the heat shock response (40).

The *E. coli* Chaperone Systems

Among the identified heat shock proteins, the GroES, GroEL, DnaK, DnaJ and GrpE proteins have been shown to function as chaperones or as parts of chaperone systems. The ClpP, ClpA and ClpX proteins are also speculated to function as a chaperone system as well (59, 67). There is some evidence indicating that some members of the heat shock regulon, such as Lon, also function as proteases in collaboration with the various chaperone systems. Recent work has indicated that there may be cooperation between chaperone systems to accomplish protein folding tasks (27). A better understanding of how these systems function is essential to understanding the protein folding problem and the means by which cells cope with stress. This section covers the experimental observations that form the basis of a model for the mode of action of the DnaK-DnaJ-GrpE chaperone system.

The DnaK-DnaJ-GrpE Machinery

Indication that the DnaK, DnaJ and GrpE proteins function together as a chaperone machine comes from *in vivo* and *in vitro* studies of the wild-type and mutant forms of these proteins. Mutations in the *dnaK*, *dnaJ* or *grpE* genes exert global effects on host cell metabolism at non-permissive temperatures including (a) RNA synthesis, (b) DNA synthesis, (c) cell filamentation, (d) lowered protease

activity, and (e) heat shock protein overproduction at all temperatures (reviewed in 15 and 19).

The *dnaK, dnaJ* and *grpE* genes can all be deleted, but only in the presence of certain suppressor mutations (45, 2, 55). Interestingly, the suppressor mutations isolated as allowing the deletion of *dnaK* will also allow the deletion of *grpE* (2). Furthermore, mutations in *dnaK* can be isolated which eliminate the need for GrpE entirely (36; Chapter 2). These results suggest that other functions can take over those of DnaK and GrpE, and indicate that GrpE has no role in *E. coli* growth and physiology other than that of regulating DnaK's activities.

DnaK. DnaK is an acidic protein with a monomeric weight of approximately 70 KDa. It has a weak ATPase activity which hydrolyzes approximately one molecule of ATP per 5 min (72). The DnaK protein is highly conserved in nature, and shares approximately 50% homology at the amino acid level with other members of the Hsp70 family of proteins. Such proteins are thought to bind to other proteins, helping them either to maintain the unfolded state, or to fold properly and/or disaggregate (reviewed in 22, 16). Consistent with this role, DnaK has been shown to be capable of interacting with many other proteins, specifically λP, casein, denatured BPTI, denatured rhodanese, denatured luciferase, σ^{32} and RNAP (33, 31, 34, 30, 27, 58, 54, 44). It has further been shown to be

capable of protecting RNAP from heat inactivation, and reactivating RNAP upon heat inactivation (58). Overproduction of DnaK in *E. coli* allows the transport of a mutant pre-lamB-lacZ fusion protein (48), and can substitute to a limited extent for the Yeast Hsc70 protein to transport pre-pro-α-factor into membranes (65). In addition, *E. coli dnaK* mutants secrete alkaline phosphatase at reduced rates (66).

The Hsp70 proteins are thought to be divided into two, distinct functional domains. The amino-terminal domain is responsible for Hsp70 family members' weak ATPase activity (13, 8, 39), while the carboxyl-terminal domain is thought to be responsible for forming stable interactions with substrates (39, 9).

DnaJ. Like DnaK, DnaJ also belongs to a large and expanding family of conserved proteins (7, 17, 57). The DnaJ-like proteins are thought to assist DnaK and other Hsp70 homologs in protein folding, and have also been found to carry out chaperone functions independently of Hsp70 (27). DnaJ has been found to bind to other proteins, such as σ^{32} and denatured rhodanese (27, 35). Consistent with its role as an aid to DnaK, DnaJ has been found to greatly enhance the ability of DnaK to bind to σ^{32} (35), and to reactivate heat inactivated RNAP (68). In support of its role as a chaperone in its own right, DnaJ has been found

to be capable of preventing denatured rhodanese from aggregating (27).

Although DnaJ's ability to interact physically with DnaK is a very weak one, observable only through cross-linking (M. Zylicz, unpublished observation), DnaJ has been found to induce a conformational change in DnaK and to act in concert with GrpE to stimulate the ATPase activity of DnaK by specifically increasing the rate of hydrolysis of DnaK-bound ATP (32, 64). Recent analysis of truncations of DnaJ indicate that such interaction with DnaK is the responsibility of the highly conserved amino-terminal domain of DnaJ (64).

GrpE. The GrpE protein is conserved in the prokaryotic world, but no eukaryotic homolog has yet been found. As mentioned above, genetic evidence indicates that GrpE functions exclusively as an assistant to DnaK.

In vitro, GrpE has been shown to interact with DnaK in a number of ways. GrpE forms a very stable, hydrophobic complex with DnaK which persists in the presence of 2M KCl, but which is disrupted in the presence of ATP (71, 37).

GrpE has been found to act with DnaJ to further stimulate the ability of DnaK to reactivate RNAP (68). GrpE has also been shown to cause the release of the λP protein from DnaK (44).

GrpE has been shown to act with DnaJ to stimulate the ATPase activity of DnaK by specifically releasing all bound

nucleotide from DnaK (32). This activity has recently been shown to have biological relevance in that mutations in *dnaK* isolated as allowing *grpE*-independent growth of *E. coli* achieve such independence in part either through increased ATPase activity or through spontaneous release of nucleotide (36, 37; Chapters 2, 3, 4 and 5).

GrpE has recently been shown to alter the conformation of DnaK in the presence of ATP (37). This activity may have biological relevance in that a DnaK mutant isolated on the basis of its ability to allow GrpE-independent growth of *E. coli* undergoes a similar change in conformation in the absence of GrpE, but in response to very low concentrations of ATP (37; Chapter 3).

The best characterized system that demonstrates the interaction between the DnaK, DnaJ and GrpE proteins is the λ DNA replication system (Fig. 1.1). In this system, the λO protein binds to the origin of λ replication (oriλ). Then, λP protein complexed with DnaB helicase of *E. coli* is attracted to and binds to λO. DnaJ binds to the oriλ–λP-DnaB-λO complex and helps "indicate" to DnaK which molecules of λP to interact with. Then, with the help of GrpE and DnaJ, and ATP hydrolysis, DnaK releases or translocates λP away from DnaB in such a way that DnaB is free to unwind λ DNA, and replication can begin (69, 70). In the λ replication system based on purified components,

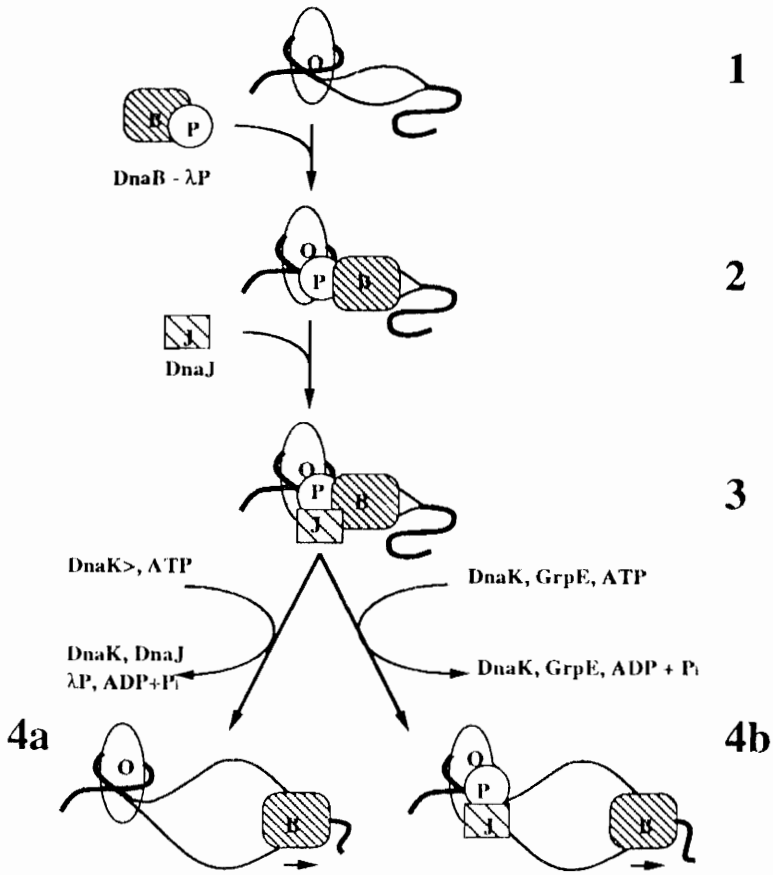

Figure 1.1. The λDNA replication system. (1) The λO protein binds to the oriλ sequences; (2) λP in complex with *E. coli* DnaB helicase is attracted to and binds to λO at oriλ, forming the preprimosomal complex; (3) DnaJ stabilizes the preprimosomal complex and tags it for recognition by DnaK; (4a) When large quantities of DnaK are present, in an ATP-dependent reaction, DnaK and DnaJ release λP from the complex allowing DnaB to unwind the λDNA thus leading to DNA synthesis; (4b) When GrpE is present, 10-fold less DnaK is required. In this case, λP is translocated within the preprimosomal complex in such a way that it no longer inhibits DnaB helicase (adapted from 69).

GrpE can be omitted provided approximately ten-fold more DnaK is added (70).

The above results coupled with the observations on λ DNA replication have led to the formulation of the following model for the DnaK-DnaJ-GrpE chaperone system. In this system, the DnaK protein binds other, mostly "unstructured" proteins and allows them either to maintain the unfolded state, or to fold and/or disaggregate. The roles of GrpE and DnaJ are to aid DnaK in both its binding and release of substrates. These tasks are accomplished by the following non-mutually exclusive functions: (a) "indicating" to DnaK which proteins are appropriate substrates, (b) maintaining a proper balance between the ATP and ADP bound forms of DnaK and (c) altering the conformation of DnaK.

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CHAPTER 2

AMINO- AND CARBOXYL-TERMINAL *dnaK* MUTATIONS CONSTITUTE DIFFERENT MEANS FOR BYPASSING THE NEED FOR *grpE*: *IN VIVO* STUDIES*

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* A. Maddock, C. Georgopoulos and D. Ang, to be submitted to J. Biol. Chem.

Abstract

It has been demonstrated that the DnaK and GrpE proteins of *E. coli* interact both *in vitro* and *in vivo*. In order to gain a better understanding of how and why these proteins interact, we have isolated and characterized single- and multicopy mutations in *dnaK* that are able to compensate for the temperature sensitive phenotype of either the *grpE*280 mutation or a *grpE* deletion. The single-copy mutants are point mutants that map to the amino-terminal end of the DnaK protein and are GrpE-independent as well as GrpE-sensitive. The multicopy mutants are truncations, from 94 to 248 amino acids, of the carboxyl-terminus of DnaK. Although each allows the growth of *E. coli* in the absence of *grpE*, none of the suppressing truncation mutants is able to support the growth of bacteriophage λ in a *grpE* mutant background, indicating that, unlike the amino-terminal mutants, they are not entirely GrpE-independent. The truncation mutants do allow the growth of bacteriophage λ in a *dnaK* mutant background, suppress the temperature sensitive phenotype and restore the heat shock response of the *E. coli dnaK*103 mutant, indicating that they retain wild-type DnaK function. However, they require a high-level expression as well as the presence of *dnaJ* to function. This work indicates that both the amino- and carboxyl-termini of DnaK are

involved in GrpE function and that mutations in the different domains of DnaK can overcome the need for GrpE by different means. This work also calls into question the size limit of the substrate interaction domain of DnaK.

Introduction

The DnaK, DnaJ and GrpE *E. coli* proteins are members of the universally conserved heat shock protein family. The rate of synthesis of these proteins is induced under stress conditions, and cells that have undergone induction of the heat shock response have been shown to exhibit increased tolerance to a variety of stresses (28). All three proteins, DnaK, DnaJ and GrpE, were originally discovered not as heat shock proteins, but rather as the products of genes, which when mutated, block the growth of bacteriophage λ at all temperatures (reviewed in 12). Both DnaJ and GrpE have subsequently been shown to interact with DnaK, either physically or catalytically (26; 20; reviewed in 14).

The DnaK protein shares approximately 50% homology at the amino acid level with members of the eukaryotic Hsp70 family (3; 21). The members of this family are thought to act by binding to other, mostly "unstructured" proteins, allowing them either to maintain the unfolded state, or to fold and/or disaggregate (35; reviewed in 14). DnaK has been shown to assist in the transport of proteins to and

through membranes (29; 9; 10; 38) and to protect and reactivate heat-denatured RNAP (35).

The DnaK protein is generally thought to be divided into two functional domains. The amino-terminal region is involved in ATP hydrolysis, and X-ray crystallographic data of the amino-terminal domain of the bovine Hsc70 protein support this conclusion (11), as do functional analyses of carboxyl-terminal deletion mutants (7; 24). The carboxyl-terminal region is thought to be involved in interaction with substrates. Although this region has not yet been crystallized, models based on the consensus for the carboxyl-terminal region of 33 members of the Hsp70 family indicate that the secondary structure of this region likely adopts a $\beta_4\alpha$ motif which is repeated twice (30). This motif is similar to and aligns well with the secondary structure of the peptide-binding domain of the HLA class I proteins (30). Furthermore, proteolytic cleavage and deletion analyses of the C-terminal ends of Hsp70 and Hsc70 have suggested that the peptide binding domain lies within the last 160, possibly up to 250, amino acids of the protein (8; 24).

The *grpE* gene shares extensive homology with genes from other prokaryotic organisms (Bin Wu, personal communication); however, no eukaryotic homolog has yet been discovered. It has previously been found that the *grpE* gene can be deleted, but only in the presence of certain

suppressor mutations (2). The suppressor mutations isolated as allowing a deletion of the *grpE* gene in all instances allow deletion of the *dnaK* gene as well (2). This finding indicates that GrpE and DnaK interact *in vivo*, and brings up the question of whether the sole function of GrpE in *E. coli* is the modulation of DnaK's activities.

The DnaK and GrpE proteins have been shown to interact *in vitro* in a number of ways. The DnaK and GrpE proteins have been shown to co-immunoprecipitate (19), and to form a very stable complex *in vitro* (39). This complex is stable in the presence of up to 2M KCl and is disrupted by the presence of ATP (39). GrpE and the DnaJ protein have been shown to jointly stimulate the ATPase activity of DnaK by approximately 50-fold (20). GrpE has also been shown to be capable of releasing both bound nucleotides (ATP and ADP; 20) and bound polypeptide (λP) from DnaK (26).

Recently, GrpE was shown to be capable of inducing a conformational change in DnaK (23). In the λ DNA replication system based on purified components, the presence of the GrpE protein reduces the requirement for DnaK by approximately 10-fold (40). In this system, GrpE works with DnaJ to help DnaK release λP from the λO-λP-DnaB protein complex which is assembled at the λ origin of replication (40; 20; 26).

In this paper, we present genetic data on two sets of *dnaK* mutations, amino- and carboxyl-terminal, which were

isolated as allowing suppression of the *grpE280* temperature-sensitive (TS) phenotype, and have been found to allow a deletion of the *grpE* gene. Two of the mutant suppressors (one amino- and one carboxyl-terminal) have been purified, and in an accompanying paper (23), biochemical data on these mutants are presented which confirm and extend the genetic findings presented in this chapter.

Materials and Methods

Strains and Media

The various bacterial strains, bacteriophages and plasmids used in this work are listed in Table 2.1. L-broth (LB) is composed of 10 g of NZ-amine, 5 g of yeast extract and 5 g of sodium chloride per liter, and the pH is adjusted to 7.4. L-agar consists of LB supplemented with 10 g of agar per liter. LB, L-agar and minimal, M-9 broth and agar (22) were used for the growth of *E. coli* strains and were supplemented with appropriate antibiotics to final concentrations of 100 μg/ml ampicillin, 20 μg/ml chloramphenicol, 50 μg/ml kanamycin, or 20 μg/ml tetracycline, or with 5 μM IPTG where necessary. P1-mediated transductions were carried out as described by Miller (25).

TABLE 2.1. Bacterial strains, bacteriophages and plasmids

Bacterial Strain, Bacteriophage or Plasmid	Relevant Characteristics	Reference or Source
Strains		
B178	W3110 *galE sup*$^+$	13
MC4100	*recA*$^+$	6
AM95	B178 *grpE*280 *pheA*::Tn*10*	1
DA100	B178 *pheA*::Tn*10* *grpE*$^+$ Ωcam^r (pDA1)	2
DA102	B178 *pheA*::Tn*10* *grpE*::Ωcam^r (pDA1)	2
AM336	C600 *dnaK*103 *thr*::Tn*10* *grpE*::Ωcam^r	2
AM483	B178 *dnaK*103 *thr*::Tn*10*	D. Ang
AM559	*dnaK52*, (= *ΔdnaK*::Ωcam^r)	27
AM299	B178 *dnaK*325	this work
AM275	*dnaK*325 *grpE*280	this work
AM266	*dnaK*325 *grpE*::Ωcam^r	this work
AM262	B178 *dnaK*332	this work
AM292	*dnaK*332 *grpE*280	this work
AM267	*dnaK*332 *grpE*::Ωcam^r	this work
Phages		
P1L4	Generalized transduction	L. Caro
λRS45	*imm*^{21} *ind*$^-$	34
λb2*c*I	clear mutant	our collection
λ*grpE*$^+$	carries *grpE* gene	31
λ*dnaK*$^+$*J*$^+$	carries *dnaK-dnaJ* operon	our collection
Plasmids		
pBR322	Amp^r Tet^r	4
pOC74	117 aa truncation of DnaK	7
pOC274	248 aa truncation of DnaK	7
pOC307	281 aa truncation of DnaK	7
pOC397	418 aa truncation of DnaK	7
pJZ514	pBR322 *dnaK* under lac promoter	J. Zeilstra
pCG1	pBR322 *dnaK*	15
pRS550	Amp^r Kan^r	34
pAM94	pCG1-derived 94 aa truncation of DnaK	this work
pAM188	pJZ514-derived 188 aa truncation of DnaK	this work

Transformations

Bacterial transformations were performed as previously described (17).

DNA Sequencing

The sequences of the *dnaK* mutations were determined by the dideoxy chain termination method (33). The required reagents and procedures were provided with the Sequenase system (United States Biochemical Corp.).

Cloning Truncations in Single Copy

Cloning of *dnaKc117*: pOC74 was partially digested with NruI, to give 6.6, 1.7, 1.4 and 0.3 kb fragments. The 1.7 kb fragment contained the *dnaKc117* gene and was ligated into the pRS550 vector (34) which had been previously digested with Bam*H1* and Eco*RI* and treated with Polymerase I. The ligation reaction was transformed into MC4100 and kan^r, amp^r, blue colonies were selected for at 37°C. The *dnaKc117* gene was then recombined onto the λRS45 bacteriophage (34), and bacteriophage carrying this gene were lysogenized into appropriate strains for characterization by standard procedures (32). Cloning of *dnaKc226*: pCG1 was digested to completion with Nru1, and the 1.4 kb fragment, which contained a truncated version of *dnaK* (whose protein product is missing 226 amino acids from

its carboxyl-terminal end), was cloned, as described above, into pRS550 and then recombined into λRS45 (34) by standard procedures (32).

Bacteriophage Burst Experiments

Bacteriophage burst experiments were performed as follows. Cultures of B178, AM95, AM299, AM275, AM266, AM262, AM292 and AM267 cells were grown overnight in LB, diluted back the following morning in LB supplemented with 0.4% maltose and allowed to grow to an OD (595 mμ) = 0.25. 1 ml of each culture was removed, infected with 10^7 λb2*cI* or λ*grpE* bacteriophage, and placed at room temperature for 30 min. Each sample was centrifuged, and the pellet washed three times with 1 ml of LB to remove any bacteriophage which had not adsorbed to the cells. 15 μl from the first supernatant of each sample were reserved, diluted and spotted on a lawn of B178 *E. coli* in order to determine the number of bacteriophage which had adsorbed. The pellets were resuspended in 1 ml of LB supplemented with 0.4% maltose and shaken in a water bath. Aliquots were removed every 15 min from 0 to 120 min, diluted and spotted on a lawn of B178 *E. coli*. The plaques were counted the following morning to determine the number of bacteriophage released from a single burst.

Heat Shock Regulation

Examination of heat shock regulation of *dnaK*-mutant cells was performed as follows. Cells were grown overnight in 2 ml of LB supplemented with appropriate antibiotics and centrifuged. The pellets were washed in 0.01M $MgSO_4$, centrifuged again and resuspended in 1 ml M9 medium supplemented with amino acids (minus cysteine and methionine). The cells were grown for 1 hr at 30°C, after which each culture was split in half, and half was placed in a tube at 30°C while the other half was placed in a tube which had been pre-warmed to 42°C. After 5 min, 20 μCi of [^{35}S]methionine per ml were added. The cells were then incubated at either 42°C or 30°C for an additional 5 min. They were then pelleted and examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography.

Western Blot Analysis

Western blot analysis was performed using the procedures and reagents provided with the ImmunoSelect kit (GIBCO BRL), and antisera to GrpE and DnaK proteins.

Results

Selection of *dnaK* Mutants

In order to gain a better understanding of how the DnaK and GrpE proteins interact, we searched for mutations

in *dnaK* which could compensate for the TS phenotype of either the *grpE*280 mutation or a *grpE* deletion (*grpE*::Ωcam^r). The *grpE* defect is characterized by two major phenotypes: (a) the cells are TS for growth at temperatures above 43°, and (b) the growth of bacteriophage λ is restricted (31; 1). We used two selections to obtain such mutations.

First, we plated the *grpE*280 strain at the non-permissive temperature, 43°C, and selected for colonies which grew well. Such colonies could have constituted (a) true revertants of the grpE280 mutation, (b) intragenic suppressors mapping within the *grpE* gene, or (c) extragenic suppressors mapping elsewhere. The isolated colonies were screened to determine which of these classes they fell into. Of the extragenic suppressors examined, approximately 80% mapped to the *dnaK* gene. Two of these mutants, *dnaK*325 and *dnaK*332 were chosen for further study.

In the other selection, we transformed plasmids pCG1 and pJZ514 that contain a copy of the *dnaK* gene under the control of either its own promoter or the *lac* promoter, respectively (both are pBR322 derivatives), into a *grpE*280 strain or one of two TS *grpE* deletion-carrying strains, *dnaK*103 *grpE*::Ωcam^r or *dnaK*332 *grpE*::Ωcam^r. We then selected for growth at the non-permissive temperature of 42°C. As a control, pBR322 with no insert was also transformed into each of the selection strains and shown

not to allow growth of the selection strains at the non-permissive temperature. Plasmid DNA was extracted from the colonies that grew at 42°C and transformed back into the strain from which it was isolated, as well as into the other two selection strains to determine (a) whether or not the mutation which allowed the strain to grow at 43°C was associated with the plasmid, and (b) whether or not mutations isolated by means of their ability to suppress the restricted growth of *grpE*280 also allowed the growth of the *grpE*::Ωcam^r strains at the non-permissive temperature and vice versa.

The *dnaK*103 mutation is a TS mutation which requires the presence of an unmapped extragenic suppressor mutation(s) in order for it to grow on rich medium (18; unpublished observations). It has been shown that the suppressor that allows the *dnaK*103 mutation also allows deletion of the *grpE* gene (2). It has also been shown that if the *dnaK*103 *grpE*::Ωcam^r strain is transduced to *dnaK*$^+$ *grpE*::Ωcam^r, the strain is still TS, demonstrating that the *grpE*::Ωcam^r mutation itself can confer a TS phenotype to *dnaK*103 *grpE*::Ωcam^r bacteria (2). Therefore, mutations in *dnaK* isolated as suppressing the TS phenotype of *dnaK*103 *grpE*::Ωcam^r would be suppressing the TS phenotype of both the *dnaK*103 mutation and the *grpE*::Ωcam^r mutation.

The *dnaK*332 mutation, in either a *grpE*$^+$ or *grpE*280 background, is TS$^+$ at 43°C (see below). This mutation in

dnaK allows a deletion of *grpE*, but only at 30°C, and at this temperature, the cells grow very slowly (see below). It is, therefore, only the presence of the *grpE*::Ωcam^r mutation in *dnaK*332 *grpE*::Ωcam^r bacteria which confers the TS phenotype. Mutations in *dnaK* isolated in this background as compensating for the TS phenotype would, therefore, be compensating for the *grpE*::Ωcam^r mutation alone.

From this selection, one pCG1-derived mutation and one pJZ514-derived mutation were obtained. Both mutations were originally isolated in the *dnaK*332 *grpE*::Ωcam^r background, and both were subsequently shown to suppress the TS phenotype of the *dnaK*103 *grpE*::Ωcam^r and *grpE*280 strains as well (see below).

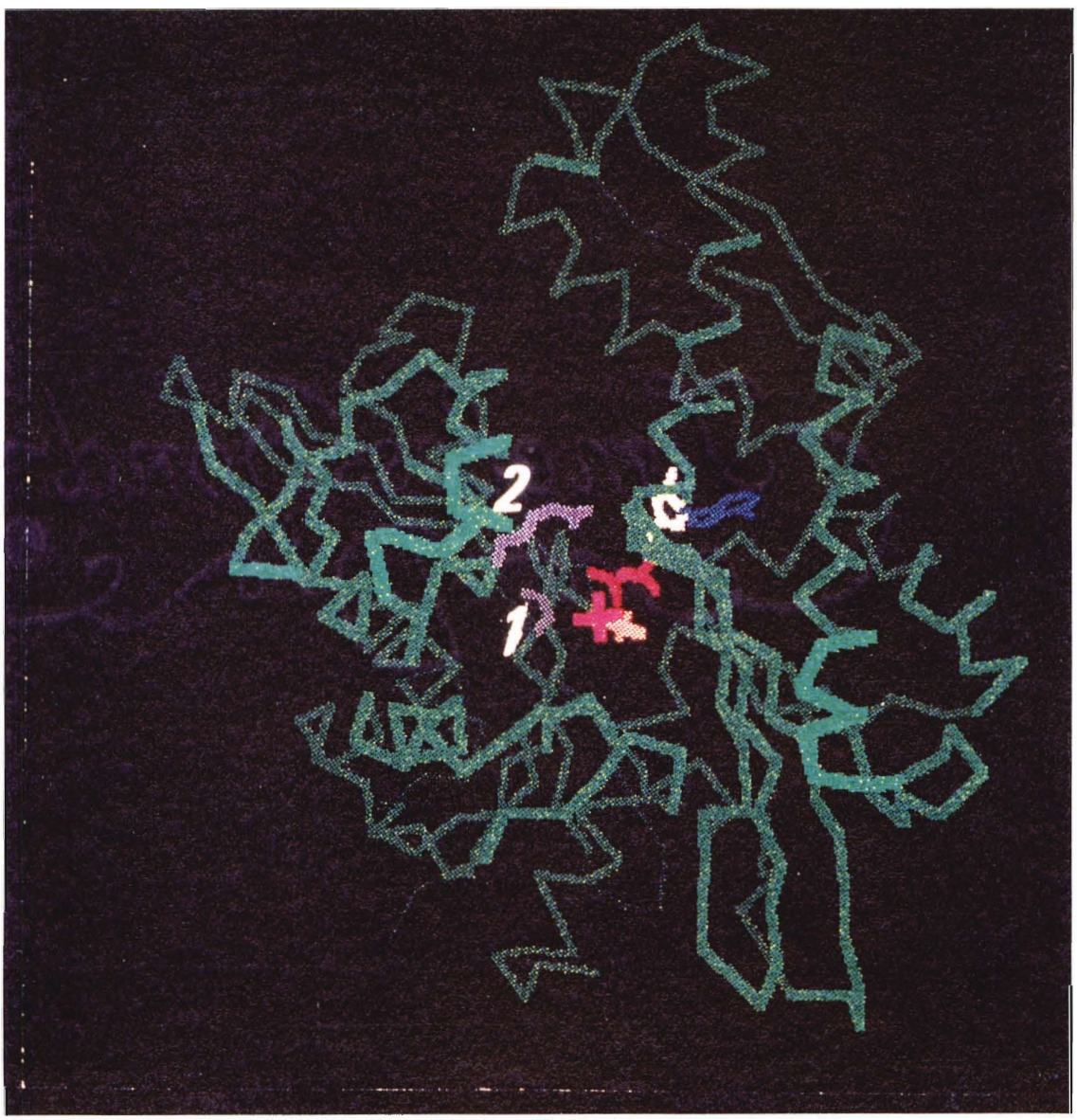
_dnaK_325 and _dnaK_332 are Amino-terminal Point Mutations

We sequenced each of the single-copy mutations in order to determine their molecular natures. Sequencing revealed that the *dnaK*325 mutation was a single point mutation from G to A at base pair 28 of the coding sequence, resulting in a change from Gly to Ser at codon 10 in the amino acid sequence. *dnaK*332 was also a single point mutation from G to T at base pair 212 resulting in a change from Arg to Leu at codon 71.

Both mutations are at conserved residues, and the residues are within highly conserved stretches of amino acids. Gly-10 is located within the highly conserved sequence Gly-Ile-Asp-Leu-Gly-Thr-Thr. Arg-71 also lies within a highly conserved stretch of amino acids, Ala-Lys-Arg-Leu-Ile-Gly-Arg-Arg-Phe (both sequences are completely conserved from DnaK to human Hsp70).

Because of the highly conserved nature of the amino acids, and because the mutations share similar phenotypes, we found it helpful to determine the exact location of these amino acid residues within the protein structure. Because of the high degree of conservation of the amino-terminal portions of the DnaK-like proteins, and because of the high degree of structural conservation seen among many ATPases (5), we assumed that the Hsc70 amino-terminal structure would represent a close approximation of the structure of the amino-terminal portion of DnaK, especially at the residues in question (11; Figure 2.1). We found that these residues are approximately 8Å from each other within the structure and are within the ATP binding site. Gly-10 corresponds to Gly-12 of Hsc70, and Arg-71 corresponds to Arg-72 of Hsc70. Lys-71 of Hsc70 has recently been shown to be directly involved in ADP binding (D. McKay and S. Willbanks, personal communication), and Asp-10 of Hsc70 has been found to be involved in Mg^{2+} and ADP binding (D. McKay and S. Willbanks, personal

Figure 2.1. Location of the *dnaK*325 and *dnaK*332 point mutations within the protein structure. (1) and (2) are the Gly and Arg residues (purple) which are mutated in *dnaK*325 and *dnaK*332 respectively. The red, white and blue molecules represent parts of the ADP and inorganic phosphate molecules. The orange molecule represents a molecule of Mg^{++}. The green line traces the α-carbon chain of the backbone. The picture is a "kinemage" generated by MAGE 1.6 written by David Richards. The Hsc70 coordinates were the kind gift of Dr. David B. McKay.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communication). That the residues that are involved in ADP and Mg^{2+} binding are so close to the mutated DnaK residues may indicate a role for GrpE in ATP binding and release from DnaK. In support of this, we have found that the DnaK332 mutant protein spontaneously releases ADP (23), and that the DnaK325 mutant protein has a very high ATPase activity (A.M., unpublished results).

_dnaK_325 and _dnaK_332 Allow Deletion of the _grpE_ Gene

In order to determine more about the interaction between the point mutants and GrpE, we tested their abilities to allow the growth of *E. coli* in the absence of *grpE*. To do this, we transduced *dnaK*325 and *dnaK*332 to tet^r at 30°C using P1 bacteriophage that had been grown on the isogenic strains DA100 and DA102 (2; Table 2.1). Both DA100 and DA102 carry a tet^r marker which is approximately 60% cotransducible with the *grpE* gene. Strain DA100 also carries an Ωcam^r cassette immediately downstream of the *grpE* gene, whereal DA102 replaces the *grpE* gene with the Ωcam^r cassette. Each of these strains also contains the pDA1 plasmid which carries a copy of the *grpE* gene under the control of its own promoter (2).

The tet^r *dnaK*325 and *dnaK*332 transductants were subsequently screened for cam^r at 30°C. Those which had been transduced to tet^r by either the P1·DA100 or P1·DA102

bacteriophage were found to have simultaneously received the cam^r marker at the expected frequencies (results not shown). The tet^r, cam^r *dnaK*325 and *dnaK*332 strains which had been transduced by the P1·DA102 bacteriophage were screened again to determine whether or not they also carried the amp^r pDA1 plasmid (which can be packaged and transferred by P1 bacteriophage). Neither set of transductants did, thus demonstrating that both *dnaK*325 and *dnaK*332 can allow deletion of the *grpE* gene.

It was of interest to us that whereas the *dnaK*325 *grpE::cam*^{r} transductants grew quickly and formed colonies of normal size, the *dnaK*332 *grpE*::Ωcam^r transductants consistently formed very small colonies which took approximately a day and a half to arise. We, therefore checked the *grpE*::Ωcam^r transductants to determine the temperature range over which they would grow, and found that while *dnaK*325 *grpE*::Ωcam^r grows well at least up to 43°C, *dnaK*332 *grpE*::Ωcam^r grows poorly, and only up to 33°C (Table 2.2). At temperatures above 33°C, this strain does not grow (Table 2.2).

grpE is poisonous to both _dnaK_325 and _dnaK_332

Because the point mutations allowed the growth of *E. coli* in the absence ofa functional *grpE* gene, we checked to see if this apparent bypass of the need for *grpE* extended to λ DNA replication as well. In order to test this, we

TABLE 2.2. Phenotypes of *dnaK* point mutants in *grpE* wild-type, mutant and deletion backgrounds.

	30°C	37°C	43°C	λb2*c*I	λ*grpE*	λ*dnaK*$^+$*dnaJ*$^+$
B178 = wild-type	+	+	+	+ (200)	+ (40)	+
***grpE*280 *dnaK*$^+$**	+	+	−	− (2)	+ (60)	−
***dnaK*325 *grpE*$^+$**	+	+	+	+ (180)	− (3)	+
***dnaK*325 *grpE*280**	+	+	+	+ (200)	− (3)	+
***dnaK*325 *grpE*::Ωcam^r**	+	+	+	+ (180)	− (2)	+
***dnaK*332 *grpE*$^+$**	+	+	+	+ (140)	− (1)	+
***dnaK*332 *grpE*280**	+	+	+	+ (188)	− (1)	+
***dnaK*332 *grpE*::Ωcam^r**	+	−	−	− (2)	+ (28)	−

+ = grows well; +/- = grows poorly; - = does not grow; ND = not determined. Strains are as listed in Table 2.1; plasmids are as listed in Table 2.1, and in the text. Numbers in parentheses indicate the approximate number of bacteriophage produced in a single burst (see text for further details).

spot tested the original isolates (*dnaK*325 *grpE*280, and *dnaK*332 *grpE*280), as well as *dnaK*325 and *dnaK*332 which had been transduced into *grpE*$^+$ backgrounds, and the *dnaK*325 and *dnaK*332 *grpE*::Ωcam^r transductants with λb2*c*I, and the transducing bacteriophages λ*grpE* and λ*dnaK*$^+$*dnaJ*$^+$ as controls (Table 2.2).

The original isolates (*dnaK*325 *grpE*280 and *dnaK*332 *grpE*280) as well as the *dnaK*325 *grpE*::Ωcam^r strain were all able to propagate λb2*c*I, indicating that the ability of *dnaK*325 to bypass the need for *grpE* extends to λ replication. The *dnaK*332 *grpE*::Ωcam^r strain was unable to efficiently plate the λb2*c*I, indicating that, as with *E. coli* growth, this mutant has some requirement for GrpE for λ replication, and that the mutant GrpE280 protein is sufficient for meeting that requirement (Table 2.2).

It was surprizing to find that neither of the *dnaK* point mutants in either the *grpE*280 or the *grpE*$^+$ backgrounds was able to plate the λ*grpE* (Table 2.2). However, when the λ*grpE*$^+$ bacteriophage was plated on these mutant strains individual plaques appeared at a frequency of 10^{-5}. Twenty-five of such plaques were purified and tested. All were found to be unable to form plaques on a *grpE*280 strain. This result indicates that the functional *grpE* gene had been lost from these mutant bacteriophages, and that it is the presence of the *grpE*$^+$ gene on the λ*grpE*$^+$

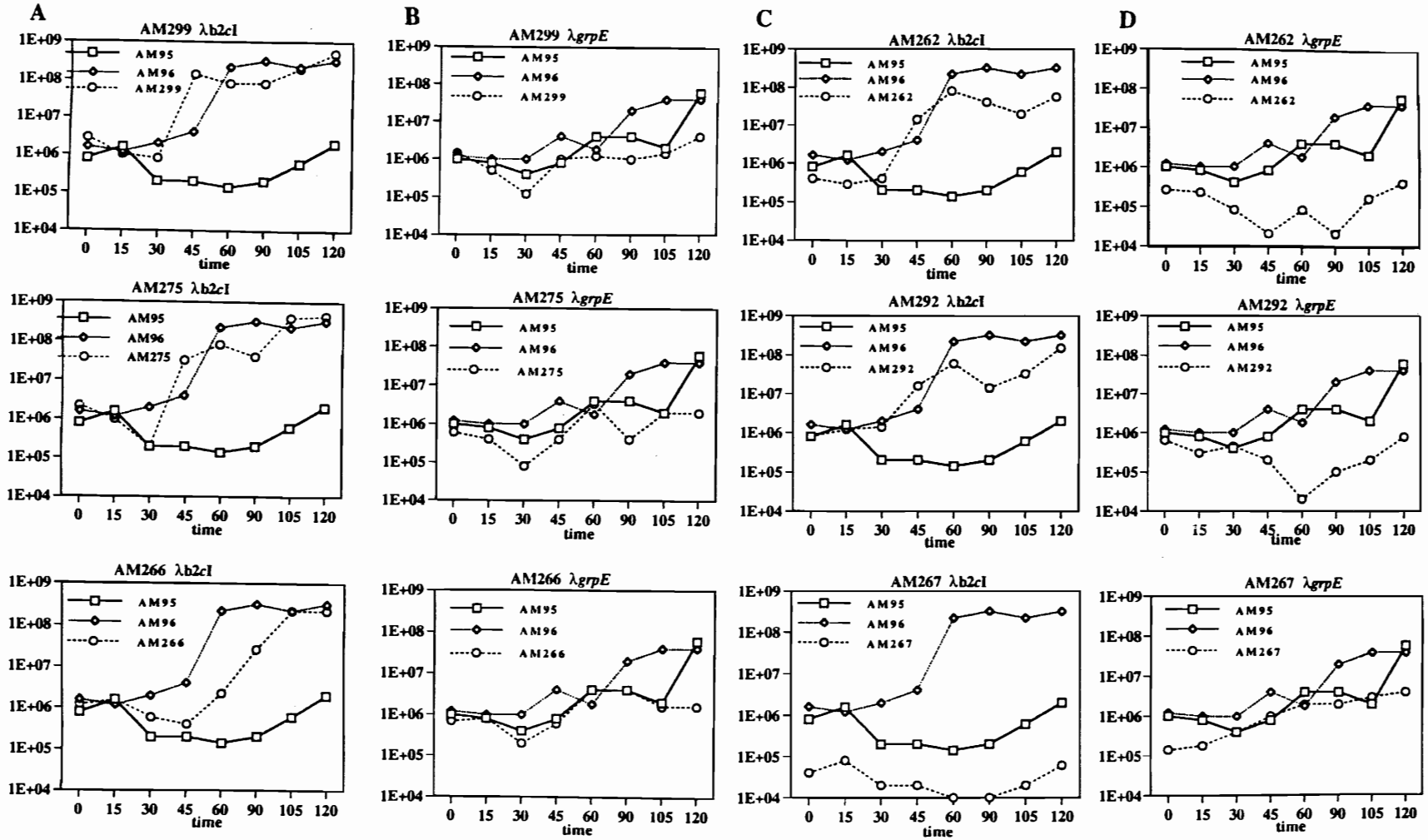
transducing phage which blocks the ability to form plaques on the *dnaK*325 and *dnaK*332 mutants.

In order to further confirm this effect and to quantitate the efficiency of the *dnaK* mutants in replicating λ, burst curves were generated for the *dnaK*325 and *dnaK*332 mutants in various *grpE* mutant backgrounds, using either λb2*c*I or λ*grpE* bacteriophages (see Materials and Methods; Fig. 2.2; Table 2.2). The number of bacteriophage produced per cell for each *dnaK*325 or *dnaK*332 strain is indicated in Table 2.2.

The results of this experiment confirm the previous findings from the spot test experiments, namely that *dnaK*325 permits the growth of bacteriophage λ in the complete absence of the *grpE* gene, that it does so nearly as well as wild-type *E. coli*, and that the presence of the *grpE*$^+$ gene on the infecting λ bacteriophage is actually highly detrimental to this activity.

The burst curve results also support our previous findings with *dnaK*332, namely that, wheras *dnaK*332 can allow the growth of bacteriophage λ in the absence of functional GrpE activity, it retains a moderate requirement for GrpE, and the mutant GrpE280 protein appears to be sufficient for carrying out this task. As with *dnaK*325, the presence of *grpE*$^+$ on a λ transducing bacteriophage is poisonous to *dnaK*332's ability to replicate λ, although,

Figure 2.2. λb2*c*I and λ*grpE* burst curves for *dnaK*325 and *dnaK*332 in various *grpE* backgrounds. Curves were generated as described in Materials and Methods. **(A)** Curves on *dnaK*325 *grpE*$^+$ (AM299), *dnaK*325 *grpE*280 (AM275) and *dnaK*325 *grpE*::Ωcam^r (AM266) bacterial strains, infected with λb2*c*I and compared with *dnaK*$^+$ *grpE*280 (AM95) and B178 wild-type (AM96) *E. coli* infected with the same bacteriophage. **(B)** Same as in (A), but the cells were infected with the λ*grpE*$^+$ transducing bacteriophage. **(C)** Curves on *dnaK*332 *grpE*$^+$ (AM262), *dnaK*332 *grpE*280 (AM292) and *dnaK*332 *grpE*::Ωcam^r (AM267) bacterial strains, infected with λb2*c*I and compared with *dnaK*$^+$ *grpE*280 (AM95) and B178 wild-type (AM96) cells infected with the same bacteriophage. **(D)** Same as in (C), but the cells were infected with the λ*grpE*$^+$ transducing bacteriophage.

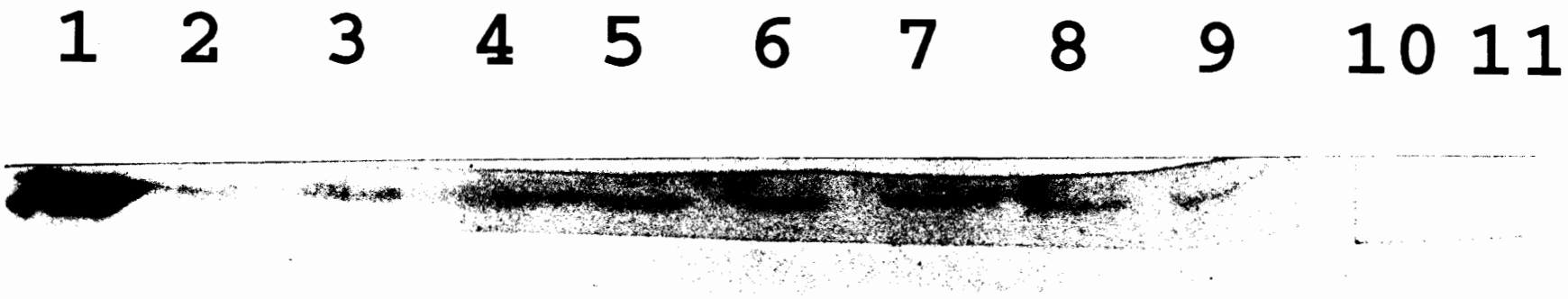

A
AM299 λb2cI
AM95
AM96
AM299
AM275 λb2cI
AM275
AM266 λb2cI
AM266
B
AM299 λgrpE
AM275 λgrpE
AM266 λgrpE
C
AM262 λb2cI
AM262
AM292 λb2cI
AM292
AM267 λb2cI
AM267
D
AM262 λgrpE
AM292 λgrpE
AM267 λgrpE
1E+09
1E+08
1E+07
1E+06
1E+05
1E+04
0 15 30 45 60 90 105 120
time

probably due to a certain level of requirement for GrpE function, this result is not as dramatic as for *dnaK*325.

That the *dnaK*325 *grpE*$^+$ and *dnaK*332 *grpE*$^+$ strains are able to replicate λb2*c*I (Table 2.2 and Fig. 2.2) indicates that a certain level of GrpE must be expressed before it interferes with DnaK325's and DnaK332's ability to function properly. In an attempt to roughly quantitate this level, we performed a Western blot analysis, using anti-GrpE sera, of extracts prepared from AM299 (*dnaK*325 *grpE*$^+$) and AM266 (*dnaK*325 *grpE*::Ωcam^r) *E. coli* which had been infected for varying lengths of time with either λb2*c*I or λ*grpE* (Fig. 2.3). The results showed that very little GrpE (no more than 2-fold excess above what is normally produced from the chromosome) is produced by the λ*grpE*$^+$ transducing bacteriophage during infection, indicating that *dnaK*325 and *dnaK*332 are extremely sensitive to the intracellular levels of GrpE.

In order to determine if the poisonous effect of GrpE which was seen for λ replication extended to *E. coli* growth as well, we attempted to bring several plasmids, both high and low copy, which contained the *grpE*$^+$ gene into the *dnaK* mutant strains and were unable to do so (results not shown). Although we were able to obtain transformants that were resistant to the drug resistance markers carried on the plasmids, each time the plasmid DNA was extracted from such transformants, and transformed back into either a

Figure 2.3. Amount of GrpE produced by a λ*grpE* phage infection. Shown is a Western blot of purified GrpE protein and cell lysates of AM299 (*dnaK*325 *grpE*$^+$; lanes 2 - 9) and AM266 (*dnaK*325 *grpE*::Ωcam^r; lanes 10 and 11) following infection with λ*grpE*$^+$ or λb2*c*I (see Materials and Methods for further details). Lane 1: purified GrpE protein; lanes 2 and 3: AM299 90 min following infection with λ*grpE*$^+$ and λb2*c*I respectively; lanes 4 and 5: AM299 60 min following infection with λ*grpE*$^+$ and λb2*c*I respectively; lanes 6 and 7: AM299 30 min following infection with λ*grpE*$^+$ and λb2*c*I respectively; lanes 8 and 9: AM299 immediately following infection with λb2*c*I and λ*grpE*$^+$ respectively; lanes 10 and 11: AM266 60 min following infection with λ*grpE*$^+$ and λb2*c*I respectively.


1 2 3 4 5 6 7 8 9 10 11

*grpE*280 or other *grpE*::Ωcam^r background, these plasmids were unable to restore the wild-type phenotype (although the parent, *grpE*$^+$-containing plasmids were able to do so). This result indicates that the plasmids isolated from the *dnaK*325 and *dnaK*332 strains no longer contained the *grpE* gene, and implies that excess GrpE may be inhibitory to the proper functioning of these point mutants in *E. coli* as we found for bacteriophage λ growth.

The Multicopy Suppressors Cause Truncations of DnaK

As a preliminary experiment to determine the nature of the multicopy suppressor mutations, cell extracts containing the *dnaK* suppressor plasmids were analyzed using SDS-PAGE. Each of the suppressor-containing extracts was unexpectedly observed to be overproducing a protein that was smaller in size than wild-type DnaK, wheras the wild-type-sized DnaK protein was not observed. The pCG1-derived mutant plasmid led to the overproduction of a protein approximately 60kD in size, and the pJZ514-derived mutant plasmid led to the overproduction of a protein approximately 50kD in size. Western blotting analysis demonstrated that the overproduced proteins are indeed truncated versions of DnaK (Fig. 2.4). Sequence analysis of the *dnaK* gene in each of the plasmids confirmed that each of the suppressors corresponded to the expected

Figure 2.4. Identification of the 94 and 188 amino acid DnaK-truncation mutants. A Western blot analysis of purified DnaK protein and various cell lysates following gel electrophoresis on a 12.5% SDS-polyacrylamide gel. Purified wild-type DnaK protein is in lane 1. Cell extracts were obtained from AM483 (dnaK103) transformed with pCG1 (lane 2), pJZ514 (lane 3), pAM94 (lane 4), and pAM188 (lane 5).

DnaK
pCG1
pUZ51A
pAM9A
pAM188

truncation of the *dnaK* gene. The pCG1-derived mutation was shown to contain an IS13 insertion (16) 283 bp from the carboxyl-terminal coding end of *dnaK* causing a 94aa truncation of the DnaK protein. The insertion extends the *dnaK* reading frame by 11 codons derived from the IS13 sequence before ending with a TAA nonsense codon (Fig. 2.5). The pJZ514-derived mutation was determined to have an A-T substitution at bp 1354 converting a Lys codon to a nonsense codon 536 bp from the carboxyl-terminal end of *dnaK*, thus causing a more severe 188 amino acid truncation of the protein (Fig. 2.5). Neither gene contains any other mutation in addition to that causing the truncation.

Up to a 248aa Truncation of DnaK Allows Suppression of the TS Phenotype of a *grpE*::Ωcam^r Strain

To extend the above finding and determine the minimum *dnaK* gene that would suppress the TS phenotype of a *grpE* mutant, we used *dnaK* truncation derivatives which had previously been constructed in our lab (7; Fig. 2.5). Four carboxyl-terminal truncations of *dnaK* were created by Bal*31* digestion of pCG1 from the Sal*1* site within the *dnaK* gene (7). Because the size of the deletions within the *dnaK* genes had previously only been estimated (7), it was necessary to determine the DNA sequences of the plasmids in

plasmid	parental plasmid	truncation caused by	~kDa	length of truncation	extra aa's	extra aa's from
pAM94	pCG1	IS13 at bp 1637	62	94	11	bp 1329-1295 IS13
pOC74	pCG1	Bal-31 bp 1652	62	117	40	bp 954-1071 pBR322
pAM188	pJZ514	A to T at bp 1354	50	188	0	--
pOC274	pCG1	Bal-31 bp 1171	50	248	57	bp 1342-1517 pBR322
pOC307	pCG1	Bal-31 bp 659	42	281	25	bp 1452-1526 pBR322
pOC397	pCG1	Bal-31	30	418	51	bp 1952-2105 pBR322

represents total aa sequence length

represents aa sequence derived from DnaK

Figure 2.5. Schematic of the *dnaK* truncation genes

order to determine the exact size of each of the truncations (Fig. 2.5).

We found that pOC74 is missing 355 bp from the 3'-end of the *dnaK* gene causing a 117 amino acid truncation of the protein. The reading frame continues using the 954 - 1074 nt from pBR322 before terminating, thus adding an additional 40 non-DnaK amino acids to the end of the truncated DnaK protein (Fig. 2.5).

pOC274 is missing 746 bp from the 3'-end of the *dnaK* gene causing a 248 amino acid truncation of the protein. The reading frame continues using the 1342 - 1517 nt from pBR322 before reaching a stop codon, thus adding another 57 non-DnaK amino acids to the end of the truncated protein (Fig. 2.5).

pOC307 is missing 846 bp from the 3'-end of the *dnaK* gene causing a 281 amino acid truncation of the DnaK protein. The reading frame, again, continues using the 1452 - 1526 nt from pBR322 before terminating, adding 24 non-DnaK amino acids to the end of the protein (Fig. 2.5).

pOC397 is missing 1258 bp from the 3'-end of the *dnaK* gene resulting in a 418 amino acid carboxyl-terminal truncation of the protein. The reading frame continues using the 1952-2105 nt from pBR322 before reaching a stop codon, adding 51 non-DnaK amino acids to the end of the protein (Fig. 2.5).

Each of these multicopy, Bal*31*-created truncations was transformed into *grpE*280, or *dnaK*103 *grpE*::Ωcam^r, or *dnaK*332 *grpE*::Ωcam^r mutant backgrounds (Table 2.3). As controls, pBR322 vector alone and the parental plasmids, pCG1 and pJZ514, as well as the two previously isolated truncations were also transformed into the above strains. The transformations were carried out both at 30°C and at 43°C. Colonies which arose at 30°C were subsequently streaked and plated at 30°C and 43°C to determine which truncations allowed suppression of the TS phenotype of the *grpE** strains. The results of this experiment are shown in Table 2.3. In summary, the 117 amino acid truncation (pOC74) was found to suppress the TS phenotype of the *grpE*280 mutation, as were the 94 amino acid truncation (pAM94) and the 188 amino acid truncation (pAM188; as previously noted). The 248 (pOC274), 281 (pOC307) and 418 (pOC397) amino acid truncations, the pBR322 vector and the parental plasmids pCG1 and pJZ514 were unable to suppress the TS phenotype of the *grpE*280 mutation. In contrast, the 117 and 248 amino acid truncations (pOC74 and pOC274) were able to suppress the TS phenotype of the two *grpE*::Ωcam^r strains, as were the 94 and 188 amino acid truncations. The 281 and 418 amino acid truncations (pOC307 and pOC397), the pBR322 vector, and the parental plasmids were unable to do so.

TABLE 2.3. Phenotypes of *dnaK* truncation mutants in *grpE*-mutant backgrounds

	30°C	43°C	λb2*c*I	λ*grpE*$^+$	λ*dnaK*$^+$*J*$^+$
AM267 = *grpE*$^\Delta$ *dnaK*332	+	–	–	–	–
AM267(pAM94)	+	+	–	–	–
AM267(pOC74)	+	+	–	–	–
AM267(pAM188)	+	+	–	–	–
AM267(pOC274)	+	+	–	–	–
AM267(pOC307)	+	–	ND	ND	ND
AM267(pOC397)	+	–	ND	ND	ND
AM267(pCG1)	+/–	–	ND	ND	ND
AM267(pJZ514)	+	–	ND	ND	ND
	30°C	**43°C**	**λb2*c*I**	**λ*grpE*$^+$**	**λ*dnaK*$^+$*J*$^+$**
AM336 = *grpE*$^\Delta$ *dnaK*103	+	–	–	–	–
AM336(pAM94)	+	+	–	–	–
AM336(pOC74)	+	+	–	–	–
AM336(pAM188)	+	+	–	–	–
AM336(pOC274)	+	+	–	–	–
AM336(pOC307)	+	–	–	–	–
AM336(pOC397)	+	–	–	–	–
AM336(pCG1)	+/–	–	ND	ND	ND
AM336(pJZ514)	+	–	–	–	–
	30°C	**43°C**	**λb2*c*I**	**λ*grpE*$^+$**	**λ*dnaK*$^+$*J*$^+$**
AM95 = *grpE*280 *dnaK*$^+$	+	–	–	+	–
AM95(pAM94)	+	+	–	+	–
AM95(pOC74)	+	+	–	+	–
AM95(pAM188)	+	+	–	+	–
AM95(pOC274)	+	–	ND	ND	ND
AM95(pOC307)	+	–	ND	ND	ND
AM95(pOC397)	+	–	ND	ND	ND
AM95(pCG1)	+	–	ND	ND	ND
AM95(pJZ514)	+	–	ND	ND	ND

+ = grows well; +/- = grows poorly; - = does not grow; ND = not determined. Strains are as listed in Table 2.1; plasmids are as listed in Table 2.1, Fig. 2.5 and in the text.

The Truncations do not Allow the Growth of Bacteriophage λ in the *grpE* Mutant Backgrounds

In order to determine whether the *dnaK* truncations that allowed suppression of the TS phenotype of the *grpE* defective strains also allowed suppression of the λ phenotype as well, we spot-tested each of the transformants (*grpE*-defective strains carrying the *dnaK* truncation suppressors) with λb2*c*I, and the transducing bacteriophages λ*grpE*$^{+}$ and λ*dnaK*$^{+}$*dnaJ*$^{+}$. The results of this experiment are shown in Table 2.3. To summarize, the *dnaK* truncations do not bypass the need for GrpE for bacteriophage λ growth suggesting that bacteriophage λ requires GrpE for growth in a *grpE* defective *dnaK* truncation suppressor background.

The Truncated DnaK's Retain Wild-type DnaK Functions

To determine genetically whether or not the *dnaK* truncations still had DnaK function, all the *dnaK* truncations were transformed into the *dnaK*103 strain at 30°C and at 42°C (Table 2.4). The *dnaK*103 mutation is a virtual deletion of the *dnaK* gene (unpublished observation), but does not exert a polar effect on *dnaJ*. This strain is TS at 42°C and is restrictive for the growth of bacteriophage λ at all temperatures (unpublished observations; Table 2.4). The 94, 117, 188 and 248 amino

TABLE 2.4. Phenotypes of *dnaK* truncation mutants in wild-type, *dnaK*- and *dnaK-dnaJ*-mutant backgrounds

	30°C	42°C	λb2*c*I	λ*grpE*$^+$	λ*dnaK*$^+$*J*$^+$
B178 = wild-type	+	+	+	+	+
B178 (pAM94)	+	+	+	+	+
B178 (pOC74)	+	+	+	+	+
B178 (pAM188)	+	+	+	+	+
B178 (pOC274)	+	+	+	+	+
B178 (pOC307)	+	+/-	ND	ND	ND
B178 (pOC397)	+	+	ND	ND	ND
B178(pCG1)	+	+	+	+	+
B178 (pJZ514)	+	+	+	+	+

	30°C	42°C	λb2*c*I	λ*grpE*$^+$	λ*dnaK*$^+$*J*$^+$
AM483 = *dnaK*103 *grpE*$^+$	+	-	-	-	+
AM483 (pAM94)	+	+	+	+	+
AM483 (pOC74)	+	+	+	+	+
AM483 (pAM188)	+	+	+	+	+
AM483 (pOC274)	+	+	+	+	+
AM483 (OC307)	+	-	ND	ND	ND
AM483 (pOC397)	+	-	ND	ND	ND
AM483 (pCG1)	+	+	+	-	+
AM483 (pJZ514)	+	+	+	+/-	+

	30°C	42°C
***dnaK52* = *dnaK*::Ωcam^r**	+	-
***dnaK52* (pAM94)**	+	-
***dnaK52* (pOC74)**	+	-
***dnaK52* (pAM188)**	+	-
***dnaK52* (pOC274)**	+	-
***dnaK52* (pOC307)**	+/-	-
***dnaK52* (pOC397)**	+/-	-
***dnaK52* (pCG1)**	+/-	-
***dnaK52* (pJZ514)**	+	-

+ = grows; +/- = grows poorly; - = does not grow; ND = not determined. Strains are as listed in Table 2.1; plasmids are as listed in Table 2.1, Fig. 2.5 and in the text.

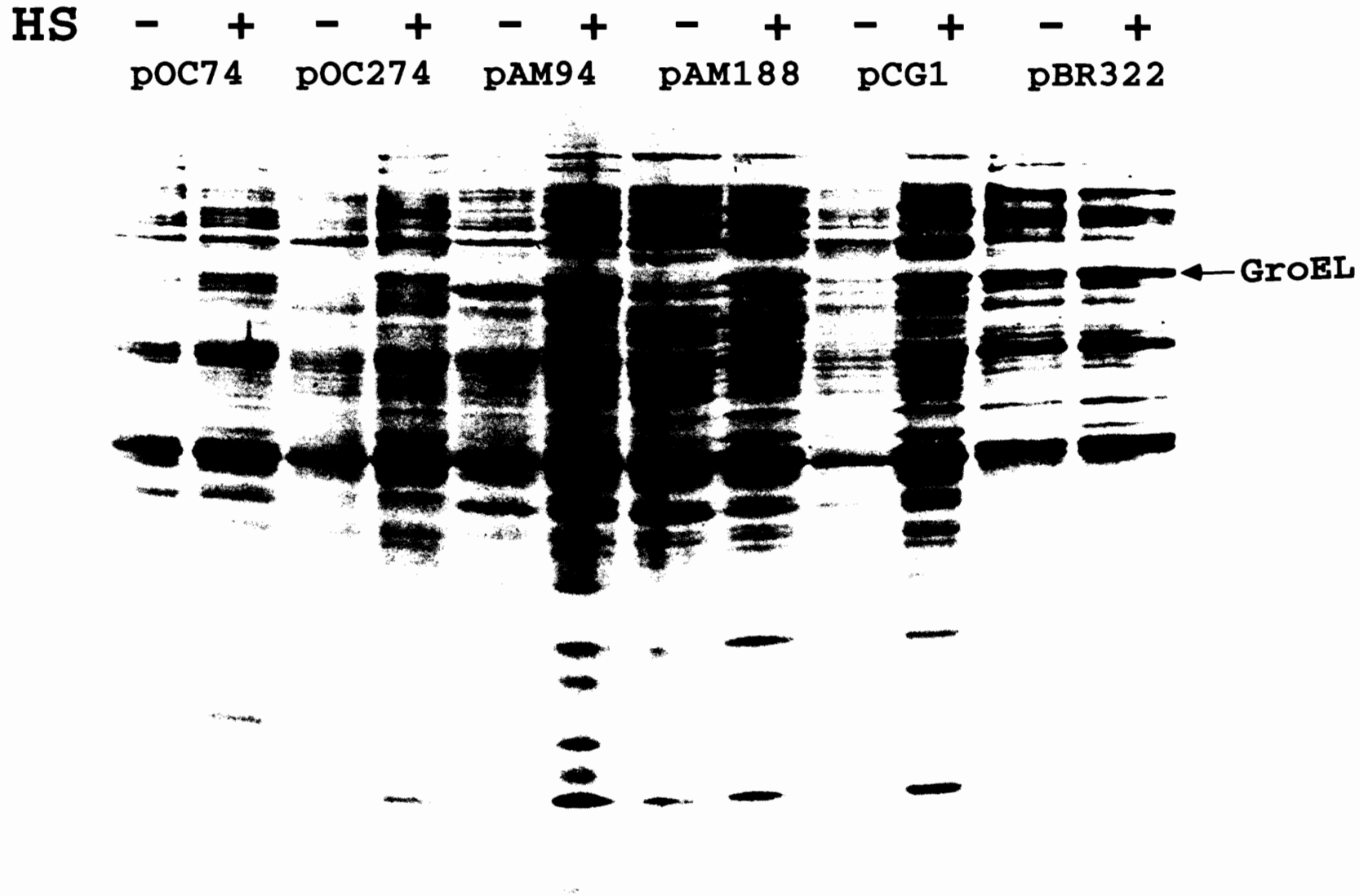
acid truncations (pAM94, pOC74, pAM188 and pOC274 respectively) were all able to suppress the TS phenotype of *dnaK*103, as were the *dnaK*$^+$ parental plasmids, pCG1 and pJZ514 (Table 2.4).

However, the 281 and 418 amino acid truncations (pOC307 and pOC397) were unable to suppress this TS phenotype (Table 2.4).

In order to determine whether the truncations allowed the growth of bacteriophage λ as well, *dnaK*103 that had been transformed with each of the TS-suppressing truncations was spot tested with λb2*c*I, λ*grpE*$^+$ and λ*dnaK*$^+$*dnaJ*$^+$. The truncations, up to 248 amino acids (pAM94, pOC74, pAM188 and pOC274), restored the ability of bacteriophage λ to plate on the *dnaK*103 strain (Table 2.4), thus indicating that they retain enough wild-type function for bacteriophage λ to replicate.

It has been observed that mutations in *dnaK* often result in a deregulation of the heat shock response. This loss of regulation results in heat shock protein overexpression at all temperatures (36; 37; 27; 19; C. Gross personal communication, Fig. 2.6). In order to determine whether the truncations that were able to suppress the TS and λ phenotypes of *dnaK*103 were also able to restore regulation of the heat shock response to this strain, *dnaK*103 strains carrying the suppressing truncation plasmids, as well as *dnaK*103 carrying pBR322 vector alone,

Figure 2.6. Effect of *dnaK* truncation suppressors on regulation of the heat shock response of *dnaK*103 mutant bacteria. Shown is an autoradiogram of ^{35}S-labeled cell lysates following SDS-PAGE on a 12.5% gel. Extracts were obtained from AM483 (*dnaK*103) transformed with pOC74 (lanes 1 and 2), pOC274 (lanes 3 and 4), pAM94 (lanes 5 and 6), pAM188 (lanes 7 and 8), pCG1 (lanes 9 and 10), and pBR322 vector (lanes 11 and 12). HS (-) indicates cells which were treated at 30°C, and (+) indicates cells which were heat shocked at 42°C (see text and Materials and Methods for further details).


HS
- + - + - + - + - + - +
pOC74
pOC274
pAM94
pAM188
pCG1
pBR322
GroEL

were grown at 30°C, pulse-labeled with ^{35}S-methionine at 30°C or heat shocked at 43°C, and their extracts were examined by SDS-PAGE and autoradiography (see Materials and Methods; Fig. 2.6). As can be seen in Fig. 2.6, the *dnaK*103 strains which carry the suppressing truncation plasmids no longer overproduce heat shock proteins at 30°C, whereas *dnaK*103 transformed with the vector alone does. This indicates that the suppressing DnaK truncations have restored the ability of *dnaK*103 to regulate its heat shock response.

The *dnaK* Truncation Suppressors do not Bypass the Need for DnaJ for *E. coli*, as they do for GrpE for *E. coli*

It has been demonstrated that the DnaK, DnaJ and GrpE proteins work in concert in a number of different functions such as bacteriophage λ replication and reactivation of RNA polymerase (40; 35). Therefore, it seems possible that a mutation in *dnaK* that allows a bypass of GrpE function for the host may allow a bypass of DnaJ function as well. This is not the case for the truncation suppressors.

The *dnaK* truncation suppressors were transformed into a *dnaK* deletion strain that has a polar effect on the *dnaJ* gene (*dnaK52*; 27; unpublished observation) to see if they were able to suppress the TS phenotype of this strain (27),

and proved to be unable to do so (Table 2.4). This result, taken together with the knowledge that the suppressors are able to suppress the TS phenotype of the *dnaK*103 mutation (a near deletion of the *dnaK* gene that does not have a polar effect on the *dnaJ* gene; unpublished observation), and that they are able to suppress the TS phenotype of strains defective in *grpE* function, indicates that the truncations do not bypass the need for DnaJ as they do the need for GrpE.

The *dnaK* Truncations must be Expressed in Greater than Single Copy to Exert their Effect

It was of interest to us that in previous attempts to identify mutations in *dnaK* that compensate for defects in *grpE*, we never isolated truncations of *dnaK* when the dnaK$^+$ gene was present either as a single copy on the chromosome or a low copy number plasmid (AM, CG and Claudia Johnson, unpublished results). We therefore asked whether the truncations had to be overexpressed in order to cause suppression of the *grpE*-defective phenotype.

In order to test this possibility, we used the vectors of Simons, et al. (1987) to clone two of the truncations in single copy and then test their abilities to suppress the TS and λ phenotypes of *dnaK*103 and *grpE*280 (see Materials and Methods). From this experiment, we determined that the

117 or the 226 amino acid truncation, generated by digesting pCG1 with Nru1, were unable to suppress the TS phenotypes of *dnaK*103 or *grpE*280, nor were they able to restore the ability of *dnaK*103 to plate λ, thus indicating that the truncations must be expressed in high copy in order to carry out wild-type DnaK functions and to bypass the need for GrpE in *E. coli*.

Discussion

The locations and natures of the *dnaK* mutations that we have isolated have several implications regarding the role and location of GrpE's interaction with DnaK. The fact that one can isolate mutations in *dnaK* that allow deletion of the *grpE* gene indicates that the sole function of GrpE is, indeed, the regulation of DnaK's activities.

We have found that mutations that allow deletion of the *grpE* gene can be isolated in both the amino- and carboxyl-termini of DnaK, thus indicating that both termini are involved in interaction with GrpE. Because one of the amino-terminal mutations, *dnaK*325, allows complete GrpE independence both for *E. coli* growth and for bacteriophage λ replication, while the carboxyl-terminal mutations allow GrpE independence only for *E coli* growth, the primary site of interaction is likely to lie within the amino-terminal domain. We envision that this primary interaction with the amino-terminal domain translates into a secondary, possibly

conformational, effect on the carboxyl-terminal domain. Indeed, purification and characterization of the 94 amino acid carboxyl-terminal truncation mutant, DnaKc94, has revealed that it is still able to interact with GrpE and does so in a fashion comparable with wild-type DnaK, indicating that it retains the binding site for GrpE (23). Furthermore, conformational analyses of this protein indicate that it adopts the conformation of wild-type DnaK when in complex with GrpE (23). That the amino-terminal amino acid substitutions in addition to allowing GrpE independence, create a situation in which small quantities of GrpE grossly interfere with such DnaK mutants' abilities to function, and the fact that they are in such close spatial proximity to one another may point directly to the GrpE interaction site. Their close proximity to the ATP binding site further supports the idea of GrpE's role in the DnaK-ATP interaction (20).

The carboxyl-terminal truncation mutants still require *dnaJ* for *E. coli* growth indicating that although DnaJ and GrpE are involved in some of the same processes, such as joint stimulation of ATPase and release of substrate, they must carry out very different functions for DnaK.

Although they are functional proteins, the carboxyl-terminal DnaK truncation mutants need to be expressed in greater than single copy in order to function, implying that they are less efficient than wild-type DnaK.

Furthermore, that such a large portion of the *dnaK* gene can be deleted, yet still code for a functional protein has significant implications regarding the site and extent of the putative substrate interaction domain.

It is generally accepted that interaction with substrate is the main function of the carboxyl-terminal domain of DnaK (30). Our finding that 248 amino acids can be deleted from DnaK and that such a protein retains the ability to allow *E. coli* growth at high temperature and can restore the heat shock regulation of DnaK-defective cells, provided that the truncated protein is overexpressed, demonstrates that the carboxyl-terminal one third of the protein is unnecessary for biological activity. This mutation results in the removal of nearly the entire predicted $\beta_4\alpha\beta_4\alpha$ structure thought to be involved in peptide binding. Our results appear to sharply contrast with findings from other laboratories that have suggested that between the last 250 to 160 amino acids of the carboxyl-terminal end of the Hsp70 class of proteins are necessary for interaction with peptide (30; 8; 24). However, such differences can be reconciled with the consideration that perhaps a stable interaction with substrate is not necessary for a functional interaction, and that perhaps the amino-terminal domain is involved in interaction with substrate as well. In support of this idea, our *in vitro* analyses of one of the truncation

mutants, DnaKc94, have demonstrated that although this protein is incapable of forming a stable interaction with λP, nevertheless, it is capable of replicating λ DNA both *in vivo* and *in vitro* (23). Clearly, ideas regarding the functional domains of DnaK need further evaluation.

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CHAPTER 3

PURIFICATION AND PROPERTIES OF THE DnaKc94 MUTANT PROTEIN

Abstract

It has been found that truncated versions of DnaK, up to 248 amino acids from the carboxyl-terminus of wild-type protein are able to compensate for the temperature sensitive phenotype of the *grpE*280 mutation, or even a deletion of the *grpE* gene, provided that the truncated DnaK products are overexpressed. In an effort to understand how such mutations bypass the need for GrpE and to gain insight into the DnaK-GrpE interaction, the 94 amino acid truncation mutant (DnaKc94) was purified, characterized biochemically and compared to the wild-type DnaK protein. This mutant allows λ DNA replication *in vitro*, and protects and reactivates RNAP upon heat inactivation, but with reduced efficiency compared to wild-type DnaK. The mutant protein interacts with GrpE in a manner similar to that of wild-type DnaK, but has an impaired ability to interact with substrates such as λP. It has a 160-fold higher ATPase activity than wild-type DnaK, and conformational analyses suggest that in the absence of GrpE, it adopts the same conformation that wild-type DnaK does when in complex with GrpE. Very likely, DnaKc94 overcomes some need for GrpE through its increased ATPase acitivity, and through a conformational adaptation.

Introduction

The DnaK, DnaJ and GrpE *E. coli* proteins have been shown to interact as a chaperone system in a number of ways (reviewed in Chapter 1); however, the reasons for these interactions are not entirely clear. Previous genetic and biochemical studies of these proteins indicated that DnaK performs the major chaperoning functions in the system (16, 20, 1). DnaK substrates are thought to interact directly with the protein's carboxyl-terminus (12, 3), and DnaK is thought to maintain the substrates in an unfolded state, or to catalyze folding, unfolding or disaggregation of the substrates through the aid of its amino-terminal ATPase activity (14, 4, 5, 19). In this system, DnaJ and GrpE are thought to make DnaK more efficient. GrpE and DnaJ have been shown to jointly stimulate the ATPase activity of DnaK through DnaJ's stimulation of the hydrolysis of ATP to ADP, and GrpE's release of bound nucleotide from DnaK (9). Recently, it has been proposed that DnaJ and GrpE may also function as discrimination factors, helping DnaK to identify and interact with appropriate substrates (13, 18).

The *dnaK*c94 mutation was isolated on the basis of its ability to compensate for the temperature sensitive (TS) phenotype of either the *grpE*280 mutant or a *dnaK-grpE* double deletion *E. coli* mutant (11, Chapter 2). This mutation therefore must be able to compensate for the loss of both DnaK and GrpE functions. Indeed, mutations that

result in truncated DnaK proteins, up to 248 amino acids, were found to suppress the TS phenotype, restore regulation of the heat shock response, and restore the ability to plate bacteriophage λ in *E. coli dnaK*103 strains, although overproduction of the mutants was required to accomplish these tasks. Such results imply that the truncations are less efficient than wild-type DnaK, but can carry out wild-type DnaK functions. However, the mutations that were found to suppress the TS phenotype of *grpE* mutations were found not to restore the ability of these strains to plate bacteriophage λ, indicating that they can only partially overcome the need for GrpE (11, and Chapter 2).

These results bring up several questions which cannot be answered directly through genetic studies. First, how is deletion of the carboxyl-terminus of DnaK able to accomplish some degree of GrpE independence? Second, can a carboxyl-terminally deleted DnaK protein still interact with substrate and carry out wild-type DnaK functions? Third, what are the functions of the different domains of DnaK? In answering such questions, a better understanding of how DnaK functions and how GrpE aids in its function can be obtained.

This chapter focuses on the purification and biochemical characterization of the DnaKc94 mutant protein in terms of its ability to carry out the various DnaK functions and to interact with GrpE and substrate. The

results of such *in vitro* studies indicate that this protein is fully able to interact with GrpE, and is able to overcome some need for GrpE through two means: (1) through an inherent increase in ATPase activity, and (2) through a conformational adaptation that mimicks the form of the DnaK-GrpE complex in response to low levels of ATP. These results also indicate that, in spite of a severely hampered ability to form stable interactions with substrate, DnaKc94 is able to carry out wild-type DnaK functions *in vitro*, but, as expected from genetic studies, does so with reduced efficiency.

Materials and Methods

Proteins

Proteins of 95% or greater purity were used. GrpE protein was purified in the following manner. *E. coli* cells containing pKLM156 were grown and lysed as described (22). GrpE was purified using the same procedure as was used for DnaK described below, except that GrpE was collected in the void volume of the ATP-agarose column, dialyzed overnight in buffer A supplemented with 1M KCl and applied to a Phenyl Sepharose column (4-B from Pharmacia) equilibrated with buffer A supplemented with 1M KCl. The column was washed with the same buffer until the all unbound protein was removed from the column. A gradient from 1M to 0M KCl in buffer A was then applied to the

column, and the eluted GrpE was concentrated on a Hydroxylapetite column, previously equilibrated with buffer A supplemented with 0.2M KCl.

DnaJ protein was purified according (20) and (23), and was the kind gift of Dr. Daniel Wall.

DnaK$^+$ and DnaKc94 were purified as previously described by Zylicz et al., 1987 (21), with the following modifications. *E. coli* cells containing either pMOB45*dnaK*$^+$ or pAMKc94 were grown and lysed as described in (22). The proteins in the cleared lysate were precipitated with 0.35g/ml ammonium sulfate, resuspended in a minimal volume of buffer A (50mM Tris/HCl pH 7.2, 1mM EDTA[1] , 10% (w/v) sucrose, 0.1g/L PMSF, 10mM β-mercaptoethanol), dialyzed overnight and loaded onto a Q-Sepharose column (1.5cm x 7cm) equilibrated with buffer A. After extensive washing with buffer A (~20 column volumes), the protein was eluted with a 0.05M to 0.4M KCl gradient (250 ml x 250 ml). Fractions from the gradient which were shown by SDS PAGE to contain DnaK or DnaK mutant protein were pooled and the proteins precipitated with 0.35g/ml ammonium sulfate. The pellet was resuspended in a minimal volume of buffer B (10mM Imidazole pH 7.0, 1mM EDTA, 10% (w/v) sucrose, 0.1g/L PMSF, 5mM β-mercaptoethanol) and dialyzed overnight in

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[1] The abbreviations used are as follows: EDTA, (ethylenedinitrilo)-tetraacetic acid; DTT, dithiothreitol; Hepes, 4-(2 hydroxymethyl)-1-piperazineethanesulfonic acid; SDS PAGE, sodium dodecyl sulphate polyacrylamide gel electrphoresis; PMSF, phenylmethylsulfonyl fluoride; PEI, polyethyleneimine; BSA, bovine serum albumin; ATPγS, adenosine 5'-O-(thiotriphosphate); RNAP, RNA polymerase; HS, heat shock.

buffer B. $MgCl_2$ was added to the dialyzed, DnaK-containing fraction to a final concentration of 10mM, and the fraction was passed through a Type 1 Heparin-agarose column (1.5cm x 12cm; Sigma) equilibrated with buffer B supplemented with 10mM $MgCl_2$. The void volume was applied directly to an ATP-agarose column (1cm x 5cm; C-8 linkage from Sigma). The column was washed with one volume of buffer B supplemented with 10mM $MgCl_2$, two volumes of buffer B supplemented with 0.5M KCl and 10mM $MgCl_2$, and two volumes of buffer B supplemented with 10mM $MgCl_2$. DnaK protein of >90% purity was eluted with two volumes of buffer B supplemented with 3mM ATP and 10mM $MgCl_2$. The pure DnaK or DnaK mutant protein was precipitated with 0.35 g/ml ammonium sulfate in the presence of 10mM EDTA, resuspended in a minimal volume of buffer C (20mM Hepes/KOH pH 7.6, 1mM EDTA, 5mM DTT and 40mM KCl) and dialyzed overnight against two changes of buffer.

λP protein was purified according to the method outlined in (17). ^{14}C-labeled λP was purified according to (8).

λ DNA Replication Assay

The *in vitro* λ DNA replication assays were performed as previously described (21).

Size Chromatography

Size chromatography on a P-60 gel filtration column was performed as described by Liberek, et al., 1991 (9). Size chromatography on P-150 was performed as previously described (10) with minor modifications as described in the text and figure legends.

HPLC Chromatography

DnaK wild-type or DnaKc94 mutant protein was preincubated in the presence or absence of GrpE and/or ATP in a buffer containing 25mM Hepes/KOH pH 7.2, 150mM KCl, 25mM NaCl, 5mM $MgCl_2$ for 30 min at 42°C. The mixture was then injected onto a size exclusion SEC 4000 column (7.5mm x 300mm from Beckman) equilibrated with the same buffer. Where stated in the figure legends, the buffer also contained 125μM ATP. The chromatography was carried out at a flow rate of 1 ml/min using a Gold HPLC system (Beckman) equipped with a diode array detector.

RNA Polymerase Assay

RNA polymerase protection and reactivation assays were performed as previously described (16).

Tryptophan Fluorescence Assay

Tryptophan fluorescence was measured as described in (2).

ATPase Assay

ATPase assays were done as previously described (9).

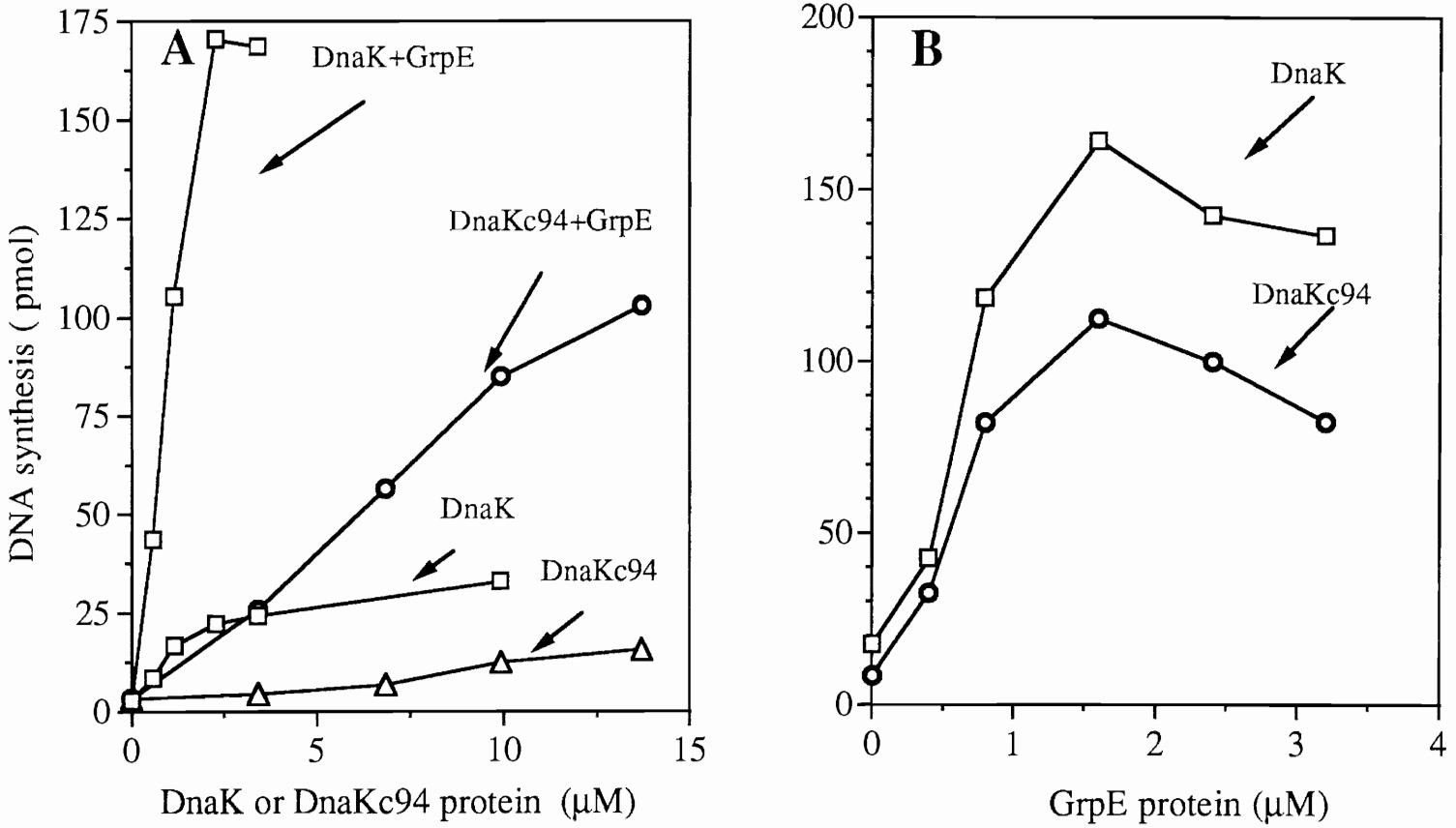
Results

λ DNA Replication Activity

In order to gain a better understanding of the molecular mechanism of GrpE's interaction with the major chaperone protein DnaK, the DnaKc94 mutant protein was purified to homogeneity. The *dnaK*c94 mutation is caused by an IS13 insertion (7) 281 bases from the end of the *dnaK* gene, resulting in a 94 amino acid truncation from the carboxyl-terminal end of the protein (11). This mutation has previously been shown to suppress the temperature sensitive phenotype of both a *grpE* deletion (*grpE::cam*^{r}) and a *grpE*280 mutant strain (11).

DnaKc94 was purified using the standard procedure elaborated for wild-type DnaK (see Materials and Methods) based on the affinity of the DnaK protein for an ATP agarose column, indicating that the mutant protein retains the ability to bind to ATP. The DnaKc94 protein was found to be partially active in the λ DNA replication system based on the crude *E. coli* protein fraction II assay (Fig. 3.1). Although DnaKc94 allows λ DNA replication, approximately 6-fold more DnaKc94 than wild-type DnaK is required to reach saturation when the fraction II extract is derived from a *dnaK*103 *grpE*$^+$ strain (results not shown).

Figure 3.1. *In vitro* λdv DNA replication by DnaK and DnaKc94. The standard replication assay premixture (21) using fraction II extracts from *dnaK*103 *grpE::cam*^{r} mutant bacteria (A) incubated with varying concentrations of DnaK or DnaKc94 with or without 1.8μM GrpE; (B) incubated with varying concentrations of GrpE and 1.14μM DnaK, or 10.7μM DnaKc94.

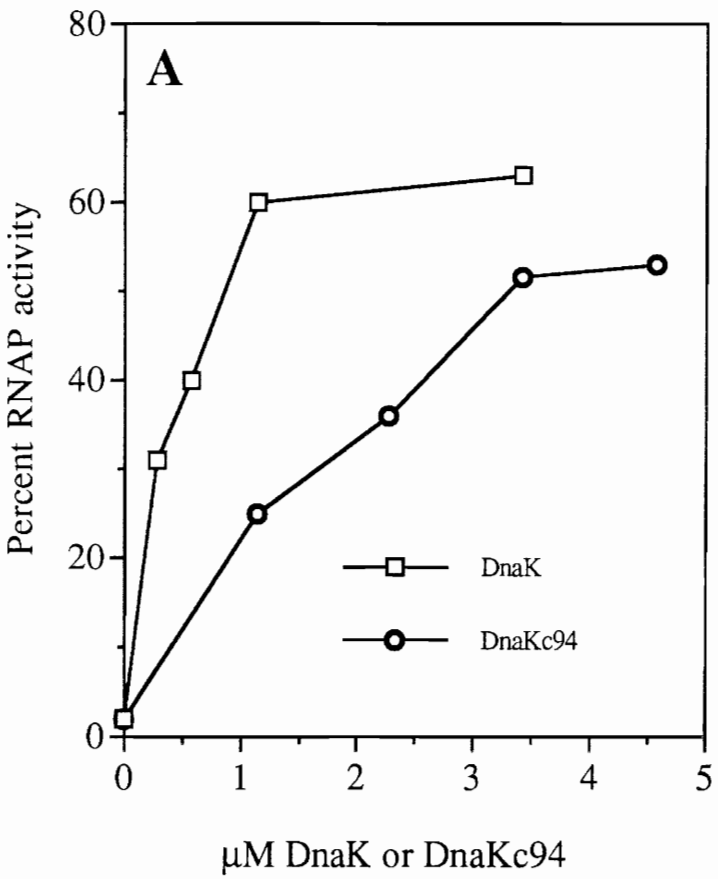

A
DnaK+GrpE
DnaKc94+GrpE
DnaK
DnaKc94
DNA synthesis (pmol)
DnaK or DnaKc94 protein (μM)
175
150
125
100
75
50
25
0
0
5
10
15
B
DnaK
DnaKc94
GrpE protein (μM)
200
150
100
50
0
0
1
2
3
4

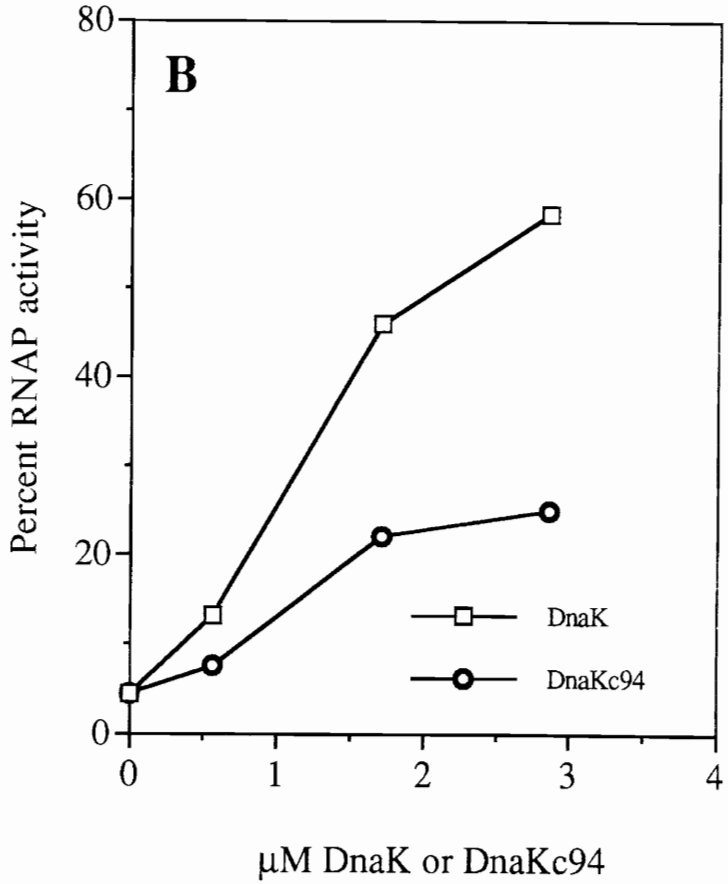
Furthermore, under these conditions, the truncated protein's saturation level for this reaction is lower than that of the wild-type protein. When a fraction II extract prepared from *dnaK*103 *grpE::cam*^{r} was used, no replication activity was seen for DnaKc94 (Fig. 3.1A), suggesting that, as was shown *in vivo*, in the case of λ DNA replication, DnaKc94 requires wild-type GrpE protein for proper activity. Indeed, the addition of GrpE to this system greatly stimulates DnaKc94-dependent λ DNA replication activity, with the optimum concentration for GrpE being in the same range as for the wild-type DnaK protein (Fig. 3.1B).

Protection and Reactivation of RNA Polymerase (RNAP) by DnaK Wild-type and DnaKc94 Proteins

It has previously been demonstrated that DnaK is capable of protecting RNAP from heat denaturation and is able to reactivate RNAP subsequent to its having been heat inactivated (16). In addition, it is known that another DnaK mutant, DnaK756, although able to protect RNAP from heat inactivation, is incapable of reactivating RNAP once it has been heat inactivated (16). The DnaKc94 truncation mutant's abilities to protect and reactivate RNAP from heat inactivation were tested, and compared with those of wild-type DnaK (Fig. 3.2).

Figure 3.2. RNAP protection and reactivation by DnaK and DnaKc94. (A) Incubation of 0.85μg of RNAP in the presence of varying concentrations of DnaK or DnaKc94 for 5 min at 30°C followed by transfer for 5 min to 50°C. Transcription assays were done at 30°C as previously described (16). (B) Heat inactivation of RNAP at 50°C for 10 min as previously described (16), followed by incubation with 2mM ATP and varying concentrations of either DnaK or DnaKc94 for 30 min at 30°C. Transcription assays were done as previously described (16). For both (A) and (B) the activity of RNAP was calculated relative to that of RNAP which had neither been treated with heat nor incubated with DnaK.


A
Percent RNAP activity
80
60
40
20
0
0
1
2
3
4
5
DnaK
DnaKc94
μM DnaK or DnaKc94


B
Percent RNAP activity
80
60
40
20
0
0
1
2
3
4
DnaK
DnaKc94
μM DnaK or DnaKc94

The DnaKc94 protein proved to be capable of both protecting RNAP from heat denaturation, and activating heat-denatured RNAP, though it was not as active as the wild-type DnaK protein (Fig. 3.2). At least three times as much DnaKc94 was required to reach saturation of protection of RNAP from heat inactivation, and the level of protection was never as high as that reached by the wild-type protein (Fig. 3.2A). Although DnaKc94 and wild-type DnaK appeared to reach saturation for the reactivation reaction at approximately the same concentrations, the truncated protein was, again, unable to reactivate RNAP to the same degree as the wild-type protein (Fig. 3.2B).

It has been shown that DnaK's ability to reactivate heat-denatured RNAP is stimulated by the presence of DnaJ and that this stimulation is further boosted by the simultaneous presence of GrpE (18). The effects of the DnaJ and GrpE cofactors on DnaKc94's ability to reactivate heat inactivated RNAP were tested. This activity was found to be stimulated by the presence of DnaJ (Table 3.1). However, unlike the wild-type protein, it was not further stimulated by the presence of GrpE (Table 3.1).

Interaction of DnaKc94 with GrpE

Because the DnaKc94 truncated protein still requires GrpE for activity for λ DNA replication, yet does not

Table 3.1 Ability of DnaJ and GrpE to stimulate DnaK's and DnaKc94's reactivation of RNAP.

	Percent RNAP Activity		Percent RNAP Activity	
Proteins		Proteins	prep 1*	prep 2*
DnaK	34	DnaKc94	27	21
+ DnaJ	47	+ DnaJ	61	50
+ DnaJ + GrpE	66	+ DnaJ and GrpE	53	43

*Preps 1 and 2 indicate DnaKc94 protein obtained from separate purifications.

require it for *E. coli* growth (11, Chapter 2) or for stimulation of RNAP reactivation, the abilities of DnaKc94 and wild-type DnaK to interact with GrpE were tested *in vitro* using size exclusion chromatography on an SEC 4000 HPLC column (Fig. 3.3).

In control experiments, the wild-type DnaK protein alone behaves on the HPLC column as if it were a globular protein with a molecular mass that is greater than the monomeric, but less than the dimeric mass of DnaK (Fig. 3.3). This suggests that, under these conditions, DnaK is forming a monomer or dimer with asymmetric shape.

When DnaK is chromatographed in the absence of ATP, a shoulder of high molecular weight protein can also be detected (Fig. 3.4). This higher molecular weight form of DnaK (which is not complexed with nucleotide; unpublished results) is not seen in the presence of ATP (Fig. 3.3). Addition of ATP to DnaK increases the absorption of the DnaK peak because of the binding of nucleotide to DnaK. In this case, free, unbound, nucleotide can also be seen (Fig. 3.4).

The GrpE protein alone behaves on the HPLC column as a tetramer (Fig. 3.3 and 3.4). This result differs from results obtained by cross-linking GrpE with glutaraldehyde where GrpE is observed predominantly as a dimer (10). When both DnaK and GrpE are mixed together in equal molar ratios, a GrpE-DnaK complex can be detected by HPLC

Figure 3.3. Interaction of DnaK and DnaKc94 with GrpE in the presence and absence of ATP. *DnaK + ATP and DnaKc94 + ATP*: DnaK (20μM) or DnaKc94 (25μM) was preincubated for 30 min at 42°C with 200μM ATP in a 40μl reaction mixture containing 25mM Hepes/KOH pH 7.2, 150mM KCl, 25mM NaCl and 5mM $MgCl_2$ and injected onto the SEC 4000 HPLC size exclusion column. The column had been equilibrated with the same buffer as above, but in the absence of ATP. *GrpE + ATP*: GrpE protein (20μM) was preincubated with ATP under the same conditions described above. *DnaK + GrpE and DnaKc94 + GrpE*: DnaK (20μM) or DnaKc94 (25μM) was mixed with GrpE (20μM) and incubated for 10 min at 42°C before being injected onto the column (no ATP was in the premixture or the column buffer). *(DnaK + GrpE)ATP and (DnaKc94 + GrpE)ATP*: The same reaction was repeated with the difference that 200μM ATP was added to both the premixture and the column buffer. In parallel with these experiments, molecular weight standards were injected onto the column under the same chromatography conditions: 1. blue dextran (Vo of the column), 2. apoferitine, (443,000) 3. β amylase (200,000), 4. BSA (68,000).

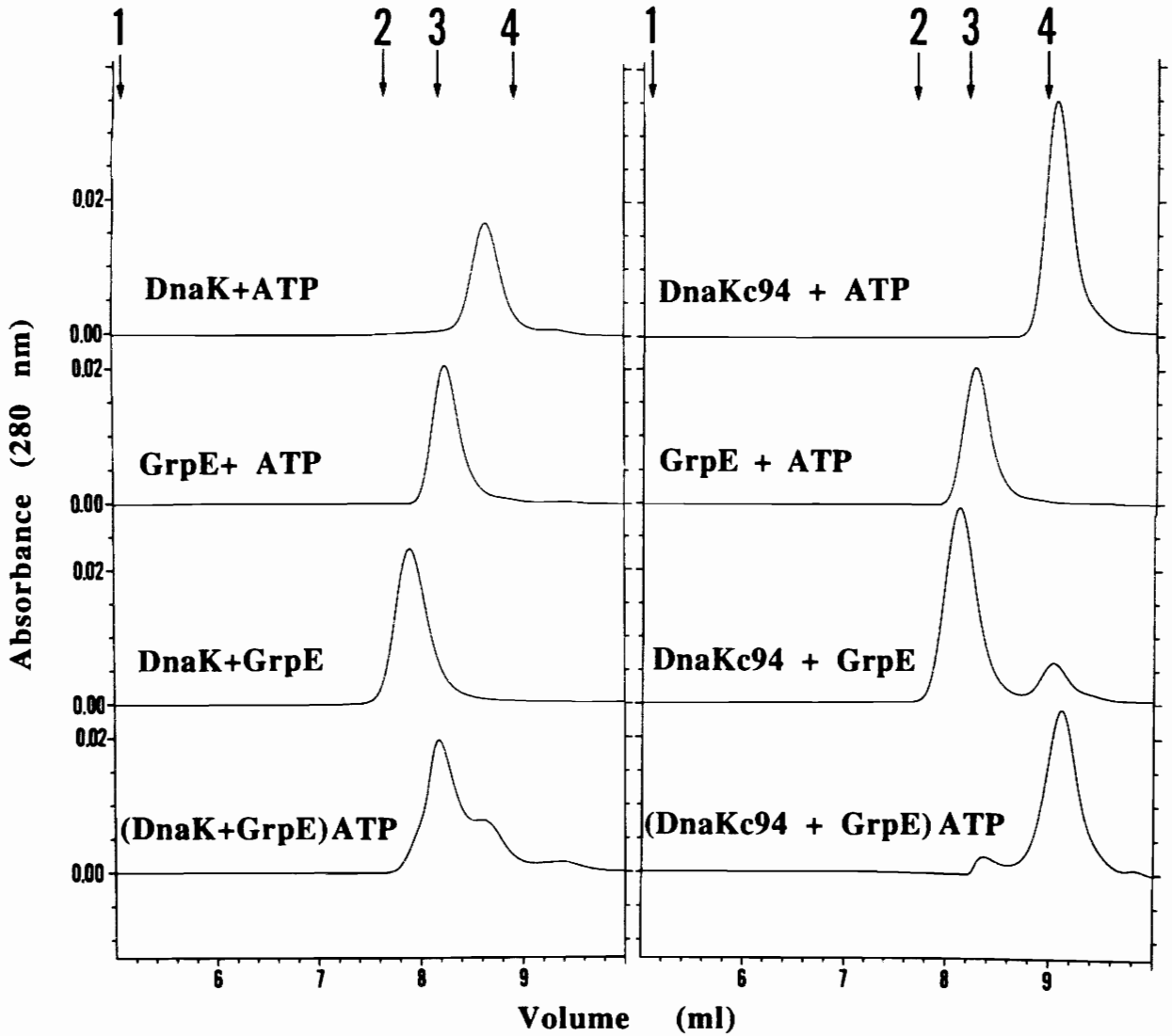
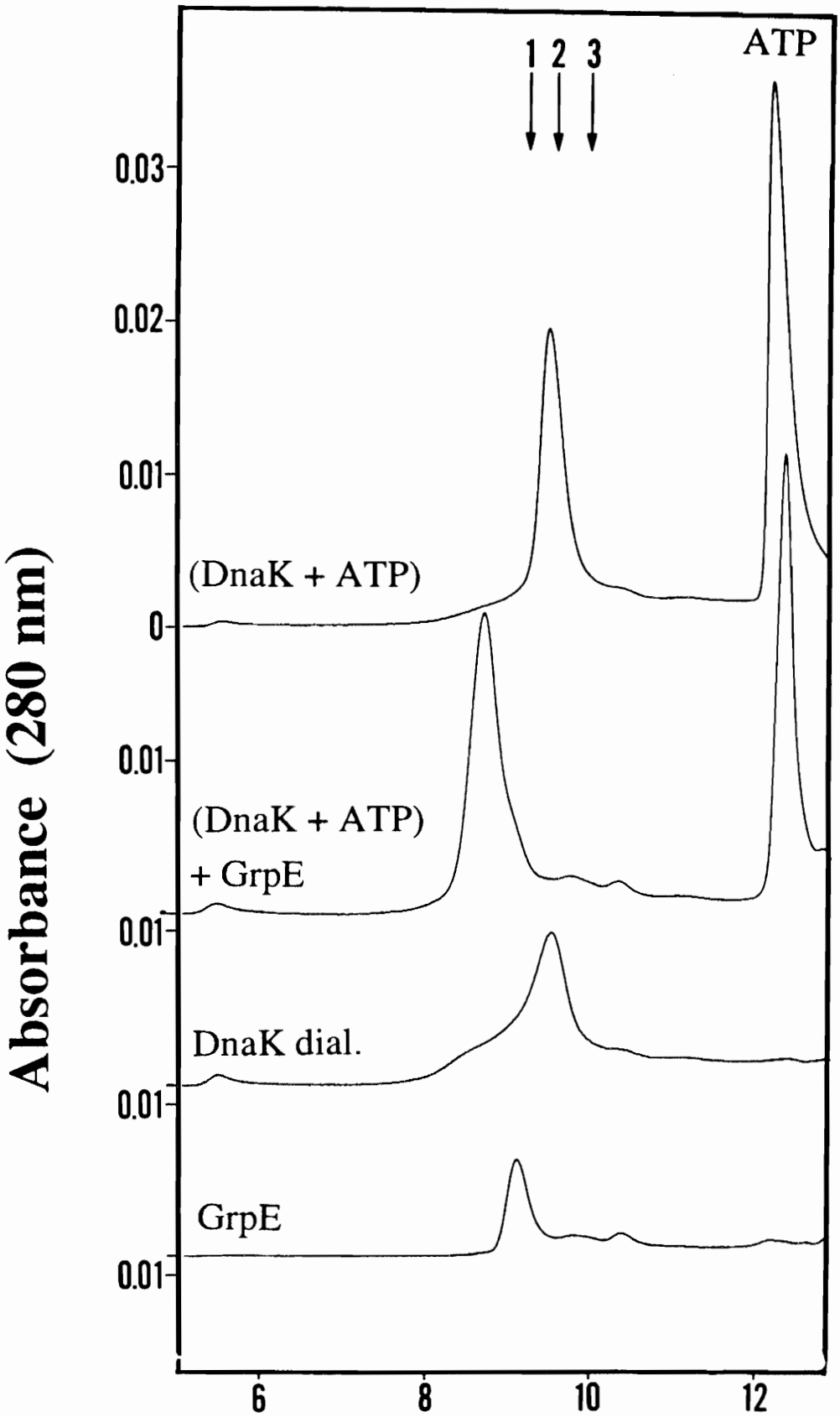

1
2
3
4
1
2
3
4
0.02
0.00
DnaK+ATP
DnaKc94 + ATP
GrpE+ ATP
GrpE + ATP
DnaK+GrpE
DnaKc94 + GrpE
(DnaK+GrpE)ATP
(DnaKc94 + GrpE) ATP
Absorbance (280 nm)
6
7
8
9
Volume (ml)

Figure 3.4. Interaction of DnaK with GrpE. The size exclusion chromatography was performed as described in the legend to Fig. 3.3 except that a guard column was applied before the SEC 4000 HPLC column. *DnaK*: 14μM DnaK after extensive dialysis in the presence of EDTA. *DnaK + ATP*: Dialyzed DnaK was preincubated with 140μM ATP for 30 min at 42°C and injected onto the column (no ATP was present in the column buffer). The position of free ATP is also seen in the chromatograph. *ATP + DnaK + GrpE*: 14μM DnaK was first preincubated with 140μM ATP for 30 min at 42°C, after which 18μM GrpE was added, and the mixture was injected onto the column (no ATP was present in the column buffer). In parallel with these experiments, molecular weight standards were applied to the column under the same conditions: 1. β amylase (200,000) 2. Phosphorylase B (94,000) 3. BSA (68,000).


1 2 3
ATP
0.03
0.02
0.01
0
(DnaK + ATP)
0.01
(DnaK + ATP)
+ GrpE
0.01
DnaK dial.
0.01
GrpE
0.01
Absorbance (280 nm)
6
8
10
12

chromatography (Fig. 3.3). Addition of ATP to only the reaction mixture does not result in dissociation of the components (Fig 3.4). The dissociation of the GrpE-DnaK complex can only be observed when ATP is present in the reaction mixture as well as in the column buffer (Fig. 3.3), suggesting that the reassociation of the DnaK-GrpE complex is a very efficient reaction which can occur even during chromatography.

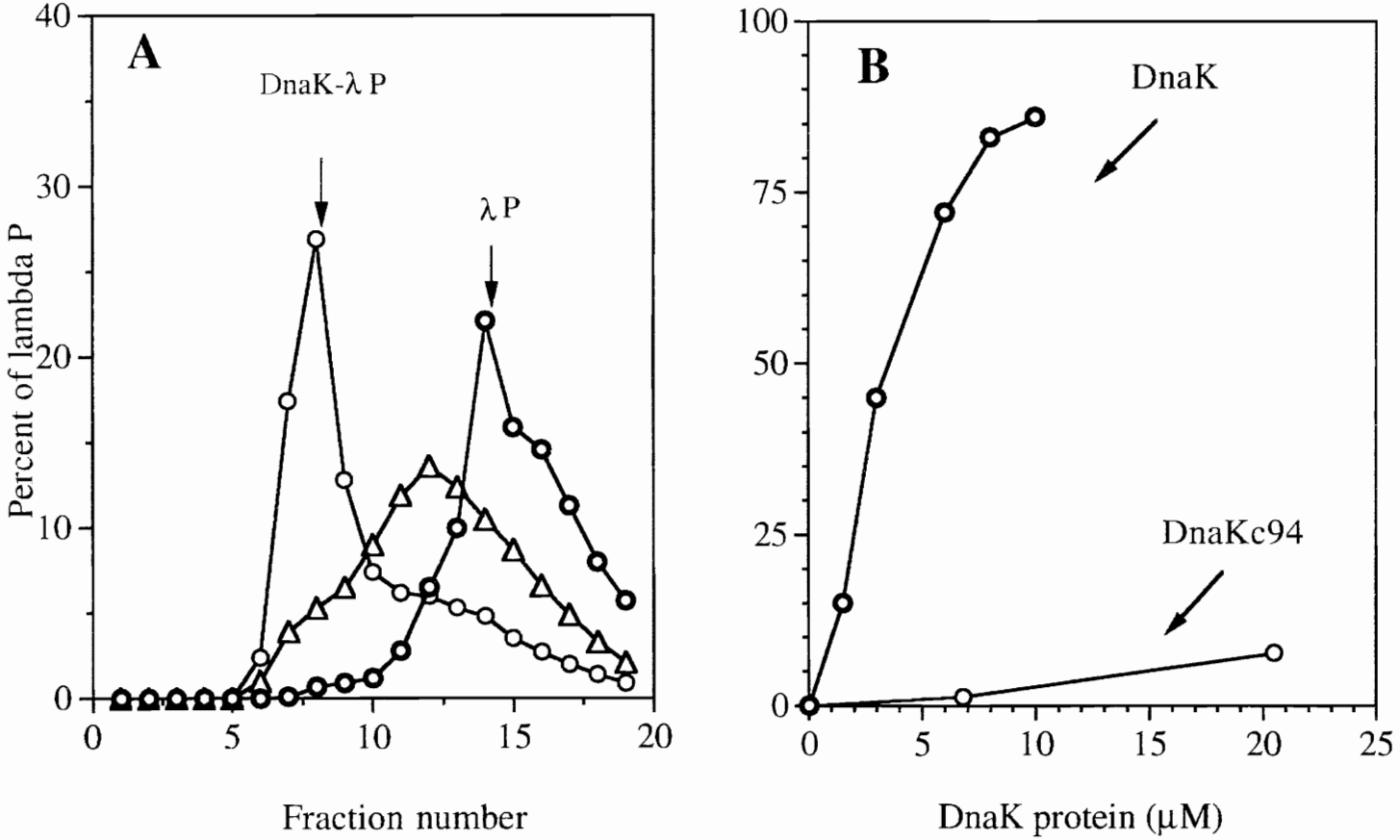
DnaKc94 (which on SDS PAGE has an apparent Mw of 60 KDa) behaves on the HPLC size exclusion column as a monomeric, globular protein with an approximate Mw of 60 KDa (Fig. 3.3). In the absence of ATP, no aggregated form of DnaKc94 is observed (results not shown), suggesting that the truncation of 94 amino acids from the carboxyl-terminal end of DnaK helps to maintain the protein in a monomeric state. DnaKc94 is able to interact with GrpE, and this complex is disrupted by the presence of ATP when it is present in both the premixture and the column buffer (Fig. 3.3). As is the case with the wild-type protein, DnaKc94 reassociates with GrpE on the column when ATP is present in the premixture but absent from the column buffer (results not shown). The results presented in Fig. 3.3 suggest that GrpE interacts with DnaK through DnaK's amino-terminal domain since the deletion of 94 amino acids from the carboxyl-terminus does not interfere with its ability to bind to GrpE.

Affinity of DnaKc94 for Substrates

In order to monitor the abilities of DnaKc94 and wild-type DnaK to bind to one of DnaK's physiological substrates, the λP replication protein, ^{14}C-labeled λP protein was purified as previously described (8) and a size exclusion chromatography assay on a P150 (BioRad) column was performed. As has previously been reported, when [^{14}C]-λP is incubated alone for 15 min at 37°C and chromatographed through a P150 column, it behaves as a monomer or dimer, eluting as a single peak in the 14th fraction (10; Fig. 3.5). The presence of increasing amounts of DnaK in the preincubation reaction alters this elution profile such that most of the [^{14}C]-λP elutes with DnaK in the void volume of the column (Figs. 3.5A and 3.5B). Also as previously demonstrated (10), the presence of ATP partially disrupts the formation of the λP-DnaK complex (Fig. 3.5A).

DnaKc94 has a much lower affinity for λP than does the wild-type protein. When a 20-molar excess of DnaKc94 over λP was present in the premixture, only 10% of the λP protein formed a stable complex with DnaKc94 (Fig. 3.5B). This result suggests that truncation of 94 amino acids from the C-terminal end of DnaK results in the loss of the ability of the protein to form stable interactions with some protein substrates. However, because DnaKc94 is at least partially active both *in vivo* (allowing *E. coli*

Figure 3.5. Interaction of DnaK and DnaKc94 with λP. Incubation of 0.75μM [^{14}C]-λP in the presence or absence of 1.71μM DnaK (A) or increasing concentrations of DnaK or DnaKc94 (B). Open triangles in panel A represent the elution profile of [^{14}C]-λP when incubated in the presence of 1mM ATP and DnaK. (B) Incubation of 0.75μM [^{14}C]-λP in the presence of increasing concentrations of DnaK or DnaKc94, and calculation of percent of [^{14}C]-λP which elutes with the DnaK or DnaKc94.


A
DnaK-λ P
λ P
Percent of lambda P
Fraction number
0
10
20
30
40
0
5
10
15
20
B
DnaK
DnaKc94
DnaK protein (μM)
0
25
50
75
100
0
5
10
15
20
25

growth and λ replication; 11) and *in vitro* (allowing λ replication and RNAP protection and reactivation; this chapter), the formation of a stable interaction between DnaK and its substrates must not be an absolute requirement for DnaK-dependent activities.

In a preliminary experiment designed to try to determine the affinity of DnaKc94 for substrates other than λP, using FPLC size chromatography, no stable interaction between DnaKc94 and α-casein was detected, though a complex between wild-type DnaK and this substrate was observed (results not shown). This result again suggests that the extreme carboxyl-terminal domain of DnaK is responsible for forming stable interactions with substrates, while rest of the molecule interacts with GrpE.

Affinity of DnaK and DnaKc94 for Nucleotides in the Presence and Absence of GrpE

The absorption spectrum (in the range of 260 to 320 nm) of DnaK which has been incubated with ATP in a 1:1, 1:10 or 1:100 molar ratio and isolated from free nucleotides by size exclusion HPLC as described in the legend to Fig. 3.3 is the super-position of two major peaks (Fig. 3.6). The 280 nm peak is derived from the absorption of aromatic amino acids, and the 260 nm peak is derived from the absorption of phosphonucleotides which are

Figure 3.6. Absorption spectra of DnaK and DnaKc94 in the presence or absence of GrpE and/or ATP. The absorption spectra of the DnaK or DnaK-GrpE complexes in the presence or absence of ATP were taken after size chromatography as described in the legend to Fig. 3.3. (A) ***DnaK dial.:*** 14μM DnaK was dialyzed extensively in Hepes buffer + EDTA. ***DnaK/ATP (1:1) (1:10) and (1:100):*** 14μM dialyzed DnaK was preincubated for 30 min at 42°C with 14μM ATP (1:1), 140μM ATP (1:10) or 1.4mM ATP (1:100) prior to injection on the column (which was equilibrated in the absence of ATP). Following chromatography, the absorption spectrum was taken using a diode array detector. (B) ***DnaK + ATP:*** 14μM dialyzed DnaK was preincubated with 140μM ATP as in (A) above. ***(DnaK + ATP) + GrpE:*** 14μM dialyzed DnaK was preincubated with 140μM ATP as in (A) above after which 18μM GrpE was added, and the mixture incubated for an additional 10 min at 42°C and injected onto the column (equilibrated without ATP). ***((DnaK + ATP) + GrpE) + ATP:*** Same as in *(DnaK + ATP) + GrpE* above except that the column was equilibrated with Hepes buffer supplemented with 140μM ATP.

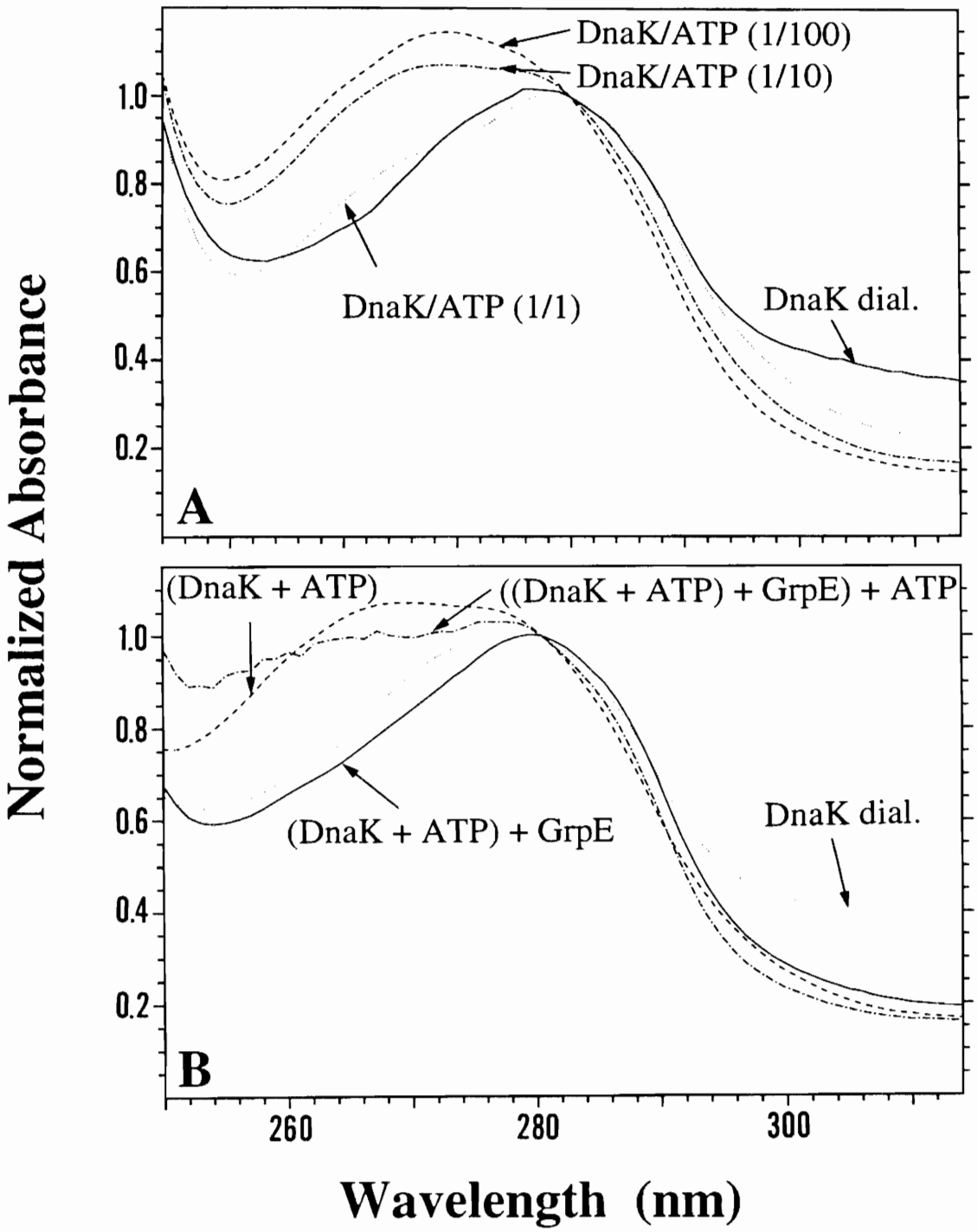
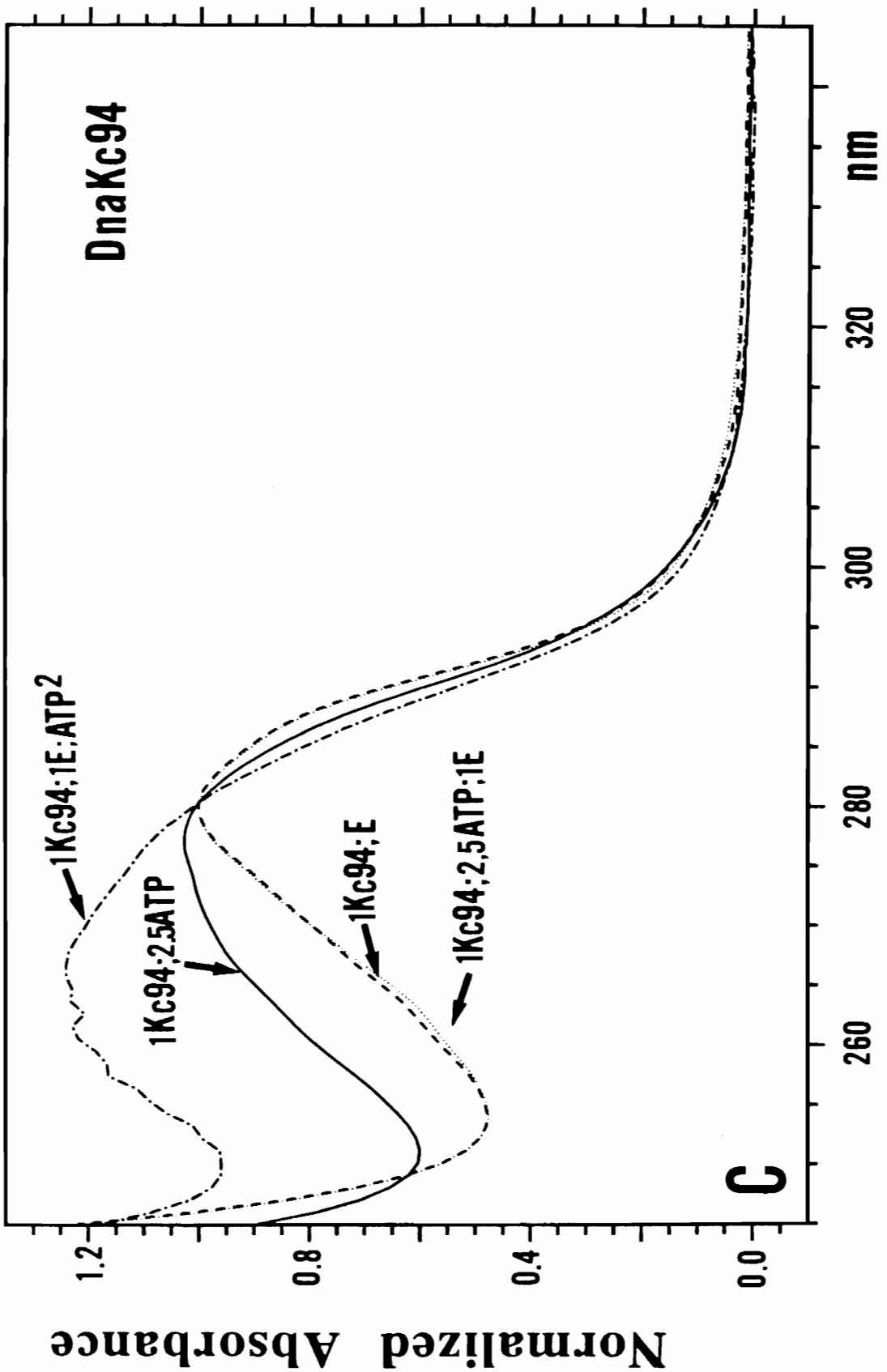

DnaK/ATP (1/100)
DnaK/ATP (1/10)
DnaK/ATP (1/1)
DnaK dial.
A
(DnaK + ATP)
((DnaK + ATP) + GrpE) + ATP
(DnaK + ATP) + GrpE
DnaK dial.
B
1.0
0.8
0.6
0.4
0.2
260
280
300
Normalized Absorbance
Wavelength (nm)

Figure 3.6C. Absorption spectra of DnaKc94 in the presence or absence of ATP and/or GrpE as in (A) and (B) above. ***1Kc94;2.5ATP:*** 14μM DnaKc94, after extensive dialysis in Hepes buffer + EDTA, was preincubated for 30 min at 42°C with 35μM ATP and injected onto the column which had been equilibrated without ATP. ***1Kc94;E:*** 14μM DnaKc94 was incubated in the presence of 14μM GrpE as described above and injected onto the column (equilibrated without ATP). ***1Kc94;2.5ATP;1E:*** DnaKc94 and ATP were preincubated as in *1Kc94;2.5ATP* above, but prior to injection onto the column (equilibrated without ATP), 14μM GrpE was added, and the mixture was incubated an additional 10 min at 42°C. ***1Kc94;1E;ATP^2:*** DnaKc94 was incubated in the presence of GrpE as in *1Kc94;E* above, but prior to injection onto the column, 35μM ATP was added, and the mixture was incubated an additional 10 min at 42°C. In this case, the column had also been equilibrated with ATP.


DnaKc94
1Kc94;1E;ATP2
1Kc94;2,5ATP
1Kc94;E
1Kc94;2,5ATP;1E
C
Normalized Absorbance
0.0
0.4
0.8
1.2
260
280
300
320
nm

bound to DnaK (Fig. 3.6A). Extensive dialysis in the presence of 10mM EDTA results in the release of most of the nucleotide from the DnaK complex and the appearance of a nearly pure protein absorption spectrum (Fig. 3.6A and 3.6B).

Addition of ATP up to a 100-fold molar excess in the premixture, before chromatography, results in only a slight increase in the 260 nm peak (as compared to the situation where only a 10-fold molar excess was used), suggesting that the addition of ATP in a 10:1 molar ratio to DnaK nearly saturates DnaK with nucleotide (Fig. 3.6A).

When DnaK is first pre-incubated with ATP in a 1:10 molar ratio, and then GrpE is added and injected onto the HPLC sizing column which has been equilibrated in the absence of ATP, a single peak is observed at the position of the DnaK-GrpE complex (Fig. 3.4). Under these conditions, the 260 nm peak disappears almost entirely, suggesting that GrpE, upon binding to DnaK, releases all bound nucleotide from it (Fig. 3.6B).

When DnaK is, again, first preincubated with ATP, and then GrpE is added and the mixture injected onto a sizing column which has instead been equilibrated with Hepes buffer supplemented with ATP, a dissociation of the GrpE-DnaK complex is observed (results not shown). The absorption spectrum of DnaK which has been separated from GrpE during this experiment posesses a significant 260nm

peak, indicating that the presence of ATP (both in the premixture and the column buffer) recycles DnaK from the complex with GrpE (Fig. 3.6B). Once it is free from GrpE, the DnaK protein is able to bind phosphonucleotide again.

The truncated protein, DnaKc94 behaved similarly to the wild-type protein, since it released nucleotide in the presence of GrpE as efficiently as DnaK (Fig. 3.6C).

In order to monitor the form of nucleotide bound to DnaK, [α-^{32}P]-ATP was pre-incubated with DnaK and the phosphonucleotide-protein complex was purified from the free nucleotides as described previously (9). This complex was then incubated in the presence or absence of GrpE and again the protein was separated from the free nucleotide using a small P-60 column. Each fraction from the second column was chromatographed on cellulose PEI plates to determine the amount of ATP versus ADP (Fig. 3.7).

As can be seen in Fig. 3.7, isolated DnaK has nearly equal amounts of ATP and ADP bound to it. As published previously, GrpE releases both ATP and ADP with equal efficiency from DnaK (9; Fig. 3.7). No difference was detected in this reaction when DnaKc94 was substituted for wild-type DnaK (Fig. 3.7), suggesting that GrpE releases nucleotide from both the DnaK and DnaKc94 mutant proteins.

As previously reported (9), the presence of DnaJ greatly stimulates the hydrolysis of the ATP which is bound to DnaK (Fig. 3.7). Because DnaKc94's rate of ATP

Figure 3.7. Effect of GrpE and DnaJ on DnaK- or DnaKc94-nucleotide complex. Incubation of 2.2μM DnaK- (column 1) or DnaKc94-[α-^{32}P]-nucleotide complex (column 2) for 1 min in the presence of 2.9μM BSA (row 1), 3.1μM DnaJ (row 2) or 2.4μM GrpE (row 3), under conditions previously described (9). A sample of each mixture was applied to cellulose PEI paper either immediately following the addition of BSA, DnaJ or GrpE (lanes A), or following the 1 min incubation (lanes B). The remaining mixture was then applied to a P-60 column, and a sample from each 2-drop fraction was applied to the cellulose PEI paper (lanes 1 - 11) for chromatography, as previously described (9).

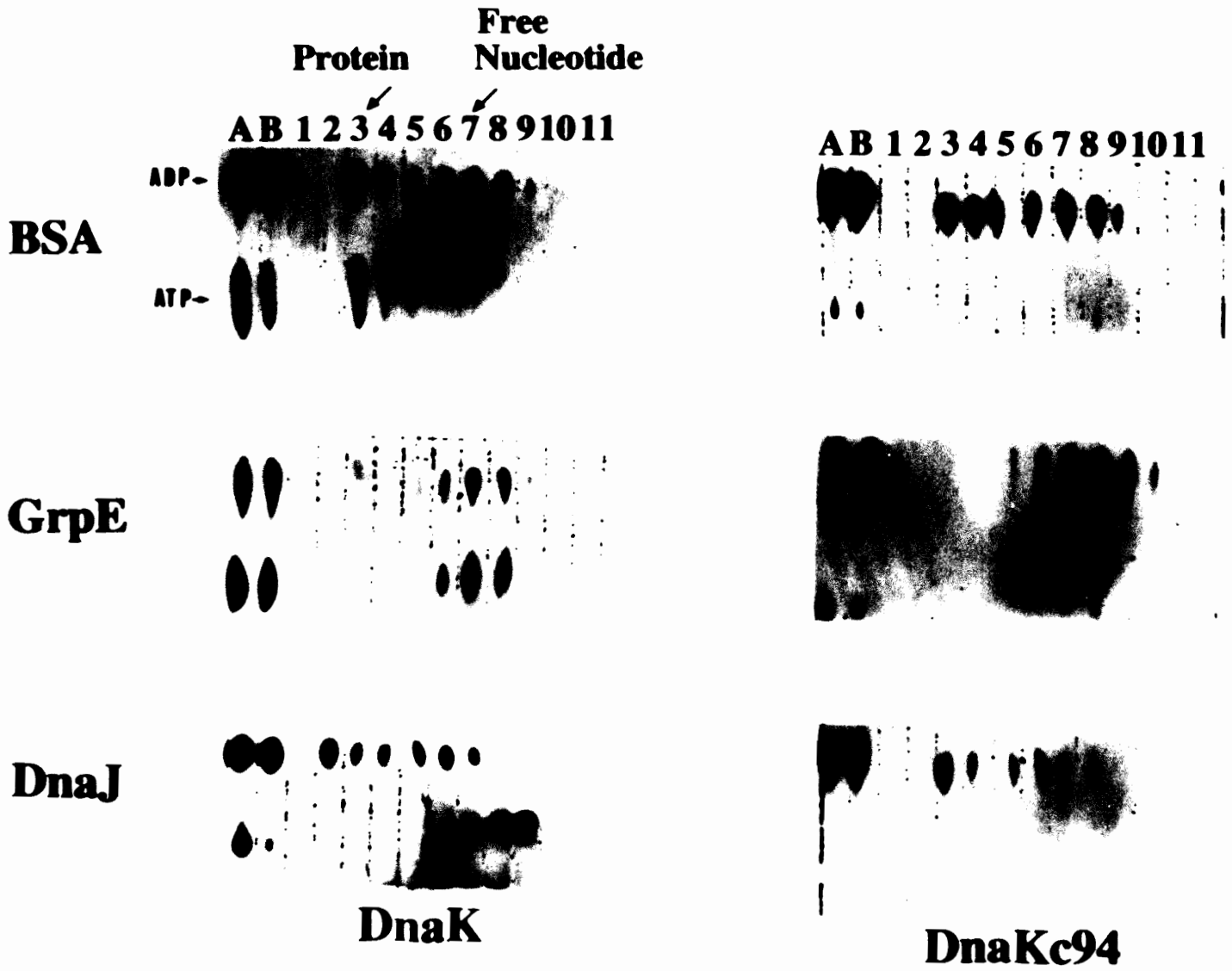
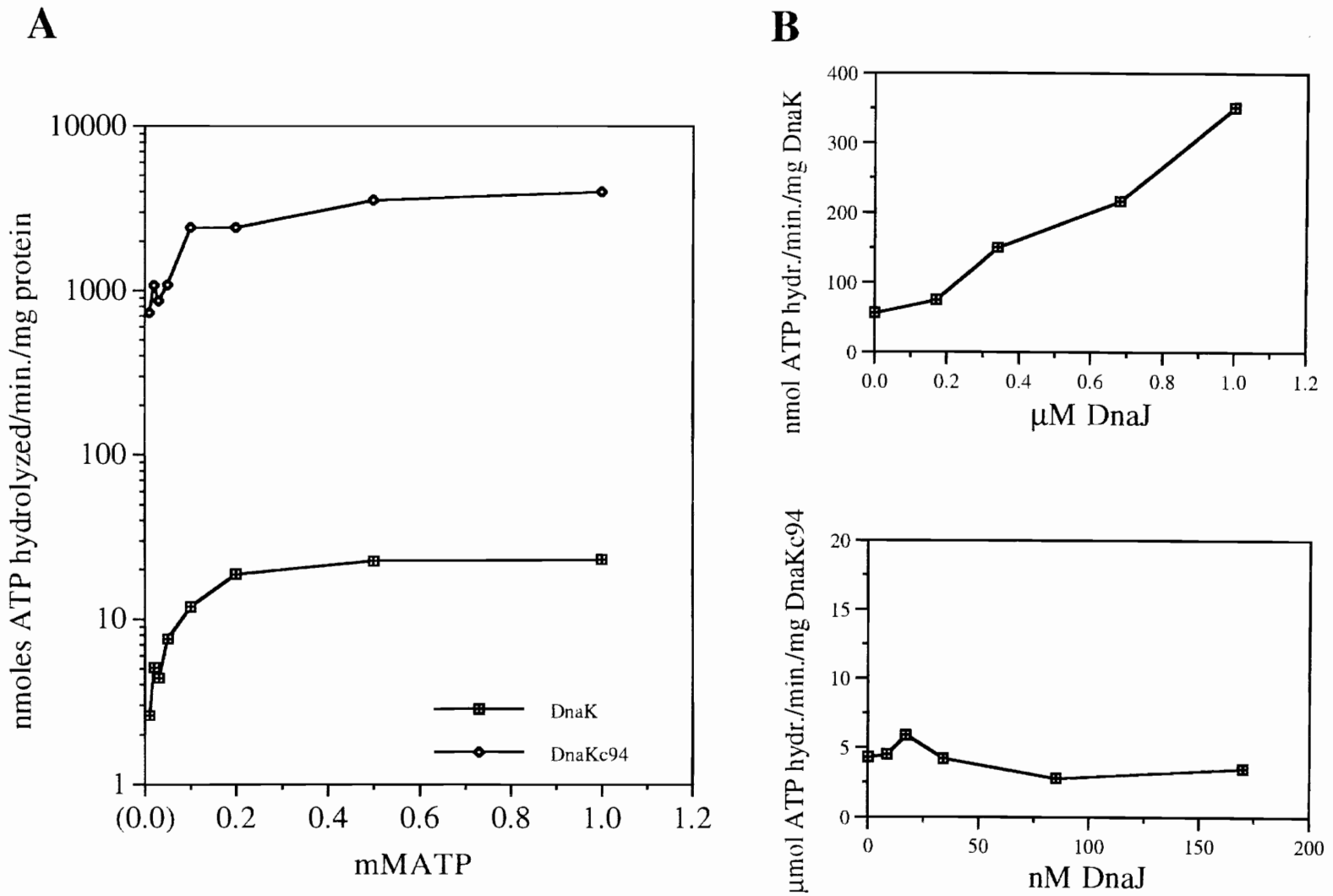

Protein
Free Nucleotide
A B 1 2 3 4 5 6 7 8 9 10 11
ADP
ATP
BSA
GrpE
DnaJ
DnaK
A B 1 2 3 4 5 6 7 8 9 10 11
DnaKc94

Figure 3.8. ATPase of DnaK and DnaKc94, and effect of DnaJ on this activity. (A) A reaction mixture of 1.7μM DnaK or 1.7nM DnaKc94 in the presence of increasing concentrations of ATP. Reaction conditions are as previously described (9). Vmax of DnaK = 38 nmoles/min/mg, Km = 0.22mM. Vmax of DnaKc94 = 6000 nmoles/min/mg, Km = 0.19mM. (B) Reaction mixtures of 0.57μM DnaK, 0.83μM GrpE and 1mM ATP or 17nM DnaKc94, 34nM GrpE and 1mM ATP with varying concentrations of DnaJ.


A
10000
1000
100
10
1
(0.0)
0.2
0.4
0.6
0.8
1.0
1.2
mMATP
nmoles ATP hydrolyzed/min./mg protein
DnaK
DnaKc94
B
400
300
200
100
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
µM DnaJ
nmol ATP hydr./min./mg DnaK
20
15
10
5
0
0
50
100
150
200
nM DnaJ
µmol ATP hydr./min./mg DnaKc94

hydrolysis is already high (Figs. 3.7 and 3.8), it is difficult to decide whether DnaJ has any further stimulatory effect on DnaKc94's rate of hydrolysis or not (Fig. 3.7).

In the control experiment presented in Fig. 3.7 (incubation of labeled wild-type or mutant DnaK with BSA), it is apparent that ADP, rather than ATP, is predominantly bound to the DnaKc94 mutant protein. This suggests that DnaKc94 has a higher intrinsic rate of ATP hydrolysis than does wild-type DnaK. Indeed, the Vmax of the ATPase activity of this mutant is approximately 160-fold higher than that of the wild-type protein, though the apparent Km remains the same (Fig. 3.8A). Moreover, when the ATPase activities for the wild-type and DnaKc94 mutant protein in complex with GrpE were assayed in the presence of increasing concentrations of DnaJ, the ATPase activity of DnaKc94 was not further stimulated by the presence of DnaJ (Fig. 3.8B). This suggests that stimulation of the ATPase activity by DnaJ is not rate limiting for this truncation mutant protein. The results presented in Fig. 3.8 clearly show that the truncation of the carboxyl-terminal domain leads to conformational changes in the amino-terminal domain of DnaK, since the ATP binding site is located in the amino-terminal domain of DnaK, yet the ATPase activity is highly affected in DnaKc94.

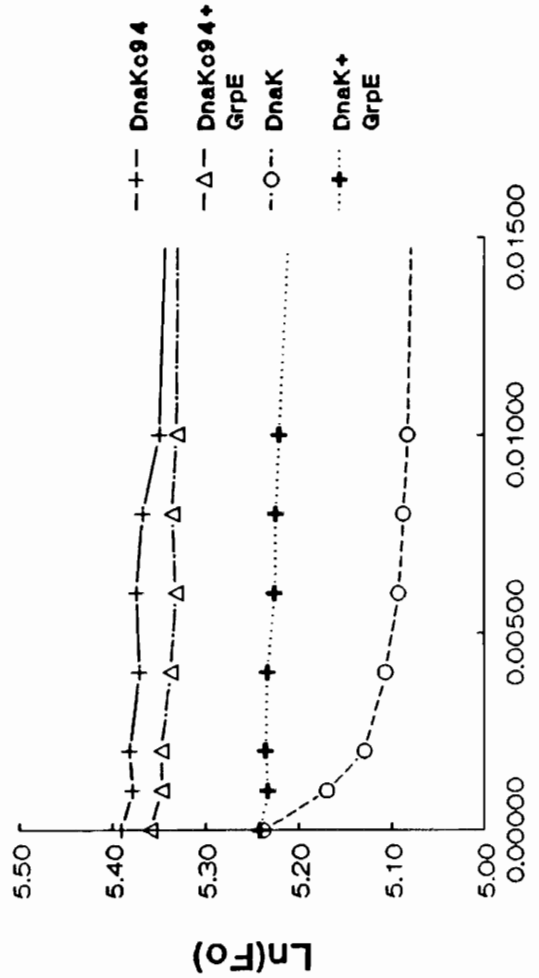
GrpE Induces a Conformational Change in DnaK

In order to answer the question of whether GrpE is able to change the conformation of DnaK, the influence of GrpE on the fluorescence of the tryptophan located in the amino-terminal domain of DnaK, near the ATP binding site was tested. The fluorescence spectra of DnaK and DnaKc94 performed in either the presence or absence of GrpE (which has no tryptophan) were almost indistinguishable from each other. However, a significant difference in the fluorescence of DnaK's tryptophan could be observed in the presence of μM concentrations of ATP (Fig. 3.9).

In the case of wild-type DnaK in the absence of GrpE, increasing the concentration of ATP quenches the tryptophan's fluorescence. When GrpE is also present (in a 1:1 molar ratio to DnaK) in this reaction, the quenching is not as efficient.

Surprisingly, the pattern of DnaKc94's tryptophan fluorescence in the absence of GrpE and the presence of ATP more closely mimicks that of wild-type DnaK in the presence of both GrpE and ATP (Fig. 3.9). The presence of GrpE does not change the quenching properties of DnaKc94 in the presence of ATP. This phenomenon cannot be explained simply by a difference between DnaK and DnaKc94 in their affinities for nucleotide in the presence or absence of GrpE since the results presented in Fig. 3.7 suggest that

Figure 3.9. Effect of GrpE and ATP on the conformation of DnaK and DnaKc94. The natural log of the flourescence intensity of the tryptophan of DnaK and DnaKc94 in the presence or absence of GrpE (in a 1:1 molar ratio to DnaK or DnaKc94) is plotted against the presence of increasing concentrations of ATP.


DnaKc94
DnaKc94+ GrpE
DnaK
DnaK+ GrpE
Ln(Fo)
5.50
5.40
5.30
5.20
5.10
5.00
0.00000
0.00500
0.01000
0.01500
[ATP] (mM)

the affinity of DnaK and DnaKc94 for nucleotide is almost the same, and that GrpE efficiently releases nucleotide from each. Instead, the results presented in Fig. 3.9 suggest that the truncation of 94 amino acids from the carboxyl-terminal end of DnaK changes the conformation of the amino-terminal domain such that it more closely mimicks the conformation of the full-length protein under the influence of GrpE.

Discussion

The mechanism by which the DnaKc94 truncated protein bypasses the need for GrpE has several implications regarding the role of the DnaK-GrpE interaction. Truncation of the carboxyl-terminal end of DnaK results in an active protein (DnaKc94), but one that is less efficient in carrying out its biological functions than the wild-type protein, since it must be overproduced in order to achieve efficient *E. coli* growth in the absence of GrpE and wild-type DnaK (11). In agreement with this interpretation, more DnaKc94 protein is required for efficient RNAP protection and reactivation, as well as for λ DNA replication in *in vitro* assays. The DnaKc94 protein has a much higher ATPase activity than does the wild-type protein in the absence of GrpE, indicating that (1) truncation of the carboxyl-terminal portion of the DnaK protein must somehow change the conformation of the amino-terminal

domain where the ATP-binding site is located; and (2) an important role for the GrpE-DnaK interaction is the stimulation of DnaK's ATPase activity, as previously predicted (9).

In spite of the fact that DnaKc94 was isolated on the basis of its ability to suppress the need for GrpE, no major differences between the ability of DnaKc94 to interact with GrpE and that of wild-type DnaK were observed. The facts that DnaKc94 is able to form a complex with GrpE observable on HPLC, that GrpE can release bound nucleotides from DnaKc94, indicate that DnaKc94 still retains a site of interaction for GrpE, and that GrpE can interact with DnaKc94. This argues that the site of interaction for GrpE is not within the last 94 amino acids of DnaK. However, the fact that *E. coli* which overproduce DnaKc94 can grow in the absence of GrpE (11), indicates that the observed interaction with GrpE does not contribute significantly to DnaKc94's biological functions.

The only difference detected between the interactions of $DnaK^+$ and GrpE versus the interaction of DnaKc94 and GrpE was the ability of DnaKc94 to change its conformation as exhibited by changes in tryptophan fluorescence in response to low concentrations of ATP. Whereas $DnaK^+$ requires the presence of GrpE to change its conformation at low concentrations of ATP, DnaKc94 is able to do so in the absence of GrpE. This finding indicates that another role

of GrpE is to induce a conformational change in DnaK and suggests that DnaKc94 may overcome the need for GrpE by adopting the conformation of DnaK which is bound to GrpE. That the conformational change in DnaK is also mediated by the presence of ATP, and that DnaKc94 can be induced to undergo the change in conformation solely by the presence of very low levels of ATP suggests that the conformational change which GrpE induces in DnaK is tied to its ability to stimulate DnaK's ATPase activity.

The question arises of whether GrpE's induction of a conformational change in DnaK is a direct effect or an indirect effect simply caused by GrpE's release of nucleotides from DnaK. DnaKc94 is able to undergo this conformational change in the absence of GrpE, stimulated only by the presence of ATP. In light of the result that DnaKc94 has an affinity for nucleotide similar to that of wild-type DnaK, this conformational change is likely not due to DnaKc94's spontaneous release of nucleotide, and argues that the effect of GrpE on DnaK's conformation is a direct one. Furthermore, were the effect an indirect one, simply due to the release of nucleotide, the incubation of DnaK in the presence of GrpE and ATP and subsequent trypsin digestion should result in a cleavage pattern that resembles that of DnaK incubated and then cleaved in the absence of ATP, which is not the case (K. Liberek and my unpublished results).

The DnaKc94 protein is not fully GrpE independent in that GrpE is still required for λ replication. Therefore, it seems likely that the GrpE-DnaK interaction serves other functions in addition to stimulating DnaK's ATPase activity and changing its conformation. It may prove to act in a manner similar to that of the DnaJ protein, that is, as a "pointer," helping to indicate to DnaK which proteins it should interact with (13). Such a scenario seems likely given DnaKc94's low affinity for λP, and its ability to replicate λ DNA only in the presence of GrpE.

In addition to their implications regarding the role of the DnaK-GrpE interaction, the results presented in this chapter have several implications regarding the functional domains of DnaK. It is generally accepted that the DnaK protein is divided into two functional domains: the amino-terminal domain is the ATPase domain, and the carboxyl-terminal domain is the site of substrate interaction. It is not surprising, therefore, that the DnaKc94 mutant protein does not bind substrate as well as does wild-type DnaK. This is likely due to the partial loss of the predicted substrate interaction domain (15, 12, 3, 6). What is surprising, in this regard, is that DnaKc94 is clearly able to form functional, if not stable, interactions with substrates, since it is a functional, albeit, less efficient, protein. This, taken together with the result that DnaK with up to 248 amino acids truncated

from its carboxyl-terminal end can remain functional (11) calls into question the site and extent of the functional interaction domain. Another implication from this work is that changes in the carboxyl-terminal end of DnaK affect the functions of the amino-terminal domain, in that truncation of the carboxyl-terminal end results in a much higher ATPase activity. This finding indicates that one responsibility of the carboxyl-terminal portion is to negatively modulate the ATPase activity of DnaK, perhaps so that its regulation can be controlled by DnaJ and GrpE.

An unexpected implication of these results regards the possible sites of interaction between the GrpE and DnaJ proteins and DnaK. Since DnaKc94 is a carboxyl-terminal truncation mutant yet can interact with GrpE in the same manner as the wild-type protein, and since DnaK332 and DnaK325 are amino-terminal mutations and have detrimental interactions with GrpE (11), it is probable that GrpE interacts with the amino-terminal domain of the DnaK protein.

The DnaKc94 protein still retains a necessary and functional interaction with DnaJ (this chapter, and 11). However, it is possible that DnaJ's ability to stimulate DnaK's ATPase activity is not coupled to its ability to help DnaK in its interaction with various substrates, inasmuch as DnaKc94's ATPase activity is not stimulated by DnaJ, yet DnaJ stimulates DnaKc94's ability to reactivate

RNAP. These results indicate that the DnaJ interaction site may be in the amino-terminal domain of the protein, possibly near the GrpE site of interaction.

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CHAPTER 4

PURIFICATION AND PROPERTIES OF THE DnaK332 MUTANT PROTEIN

Abstract

The *dnaK*332 mutation was selected based on its ability to compensate for the temperature sensitive phenotype of the *grpE280* mutation. Subsequent genetic analyses indicated that the presence of wild-type GrpE is not only unnecessary, but possibly detrimental to proper functioning of the DnaK332 protein both for bacteriophage λ replication and possibly *E. coli* functions as well. Purification and biochemical characterization of DnaK332 demonstrate that this is indeed the case. The results presented in this chapter suggest that the DnaK332 protein overcomes the need for wild-type GrpE through its spontaneous release of ADP, and that GrpE may act as a "trigger" factor for DnaK, responsible for maintaining a proper balance between DnaK which is bound to ATP and DnaK which is bound to ADP. In addition, DnaK332 may function independent of DnaJ as well.

Introduction

The *dnaK*332 mutation was isolated as an extragenic suppressor of the temperature sensitive (TS) phenotype of the *grpE*280 mutation (8, Chapter 2). The mutation was sequenced and found to cause a change in the amino acid sequence of the gene product from Arg to Leu at position 71. Further genetic characterization of the mutant demonstrated that presence of the *dnaK*332 mutation on the

bacterial chromosome allows complete deletion of the *grpE* gene up to 33°C and restoration of the ability of *grpE*280 strains to plate bacteriophage λ. However, *dnaK*332 *grpE*280 mutant bacteria were found to be unable to plate bacteriophage λ which carried a copy of the *grpE* gene (8, Chapter 2). These results indicate that although the *dnaK*332 mutation allows *E. coli* cells to grow in the absence of GrpE, it does not function completely independent of GrpE and still requires some GrpE in order to grow at high temperature. However, the mutant GrpE280 appears sufficient to allow growth at high temperature. These results further imply that the DnaK332 protein is extremely sensitive to the levels of GrpE and is "poisoned" by small amounts.

The GrpE protein has been shown to influence two aspects of DnaK's function, its ATPase activity, and its interaction with substrates (5, 9, 12). GrpE is thought to act in concert with DnaJ to stimulate DnaK's ATPase activity through its ability to release bound nucleotide from DnaK (5). GrpE has also been shown to release λP protein from DnaK (9) and is proposed to act as a "pointer" in helping DnaK to better locate its proper substrates (9, 12). Therefore, a DnaK mutant such as DnaK332, which bypasses, or at least partially bypasses the need for GrpE, should have alterations in one or both of the above functions.

This chapter deals with the biochemical characterization of the DnaK332 mutant protein with emphasis on its ability to replicate λ DNA *in vitro* in the presence and absence of GrpE, its ability to interact with GrpE, its affinity for nucleotide, and its interaction with λP. The results of these studies indicate that DnaK332 may overcome the need for GrpE through its ability to spontaneously release ADP, and may be poisoned by GrpE through GrpE's release of ATP. This, in turn, implies that GrpE may function as a "trigger factor" for DnaK, responsible for maintaining a proper balance between DnaK-ATP and DnaK-ADP. In addition, evidence is presented that the *dnaK*332 mutation allows the gene product to function independent of DnaJ.

Materials and Methods

Proteins

Proteins of 90% or greater purity were used. GrpE protein was purified as described in Chapter 3.

DnaJ protein was purified according to (14) and (17), and was the kind gift of Dr. Daniel Wall.

The purifications of DnaK$^+$ and DnaK332 were done as previously described (16), with the modifications described in Chapter 3. DnaK332 protein was isolated from *E. coli* cells containing a single copy of *dnaK*332 on the bacterial chromosome which were grown and lysed as described in (15).

λP protein was purified according to the method outlined in (10). ^{14}C-labeled λP was purified according to (4).

λ DNA Replication Assay

The *in vitro* λ DNA replication assays were performed as previously described (16).

Size Chromatography

Size chromatography on a P-60 gel filtration column was performed as described by Liberek, et al, 1991 (5). Size chromatography on P-150 was performed as previously described (6) with the minor modifications as described in the text and figure legends. HPLC was performed as described in Chapter 3.

ATPase Assay

ATPase assays were done as previously described (5).

Trypsin Digestion Assay

Trypsin digestion assays were done as previously described (7).

Results

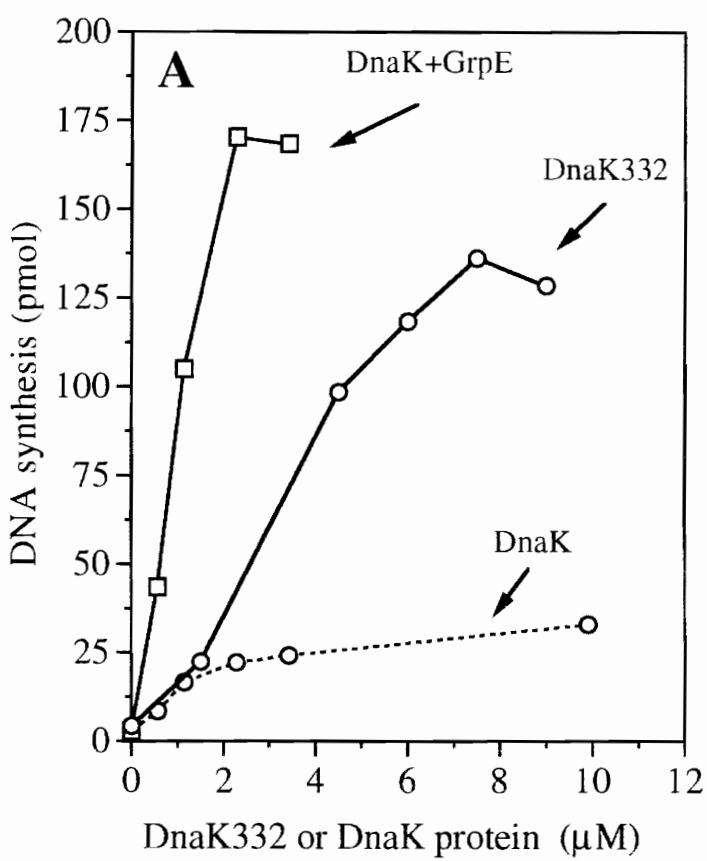
λ DNA Replication Activity

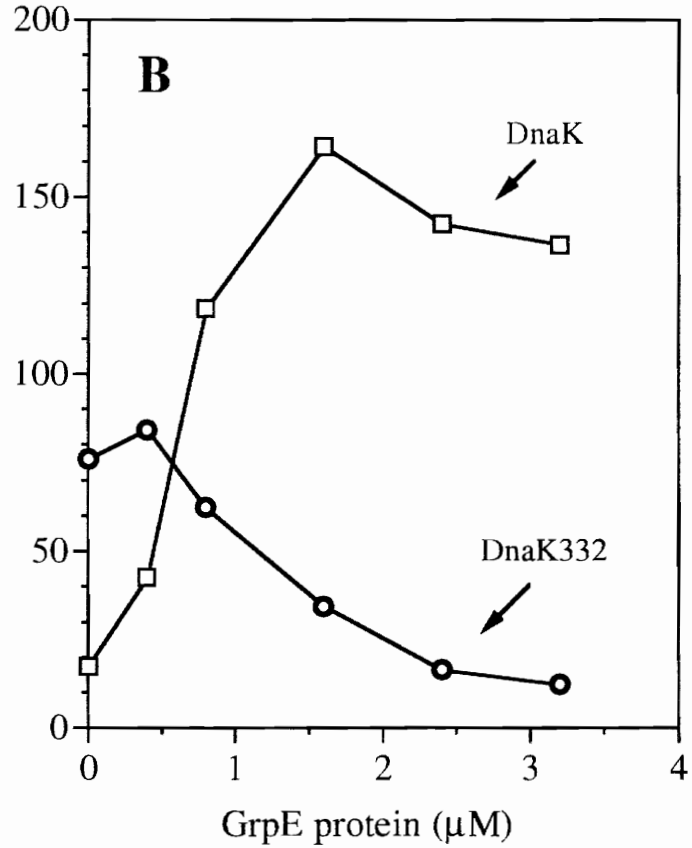
In order to gain a better understanding of the molecular mechanism of GrpE's interaction with the major chaperone protein DnaK, DnaK332 mutant protein (8) was purified to homogeneity, characterized biochemically and compared with wild-type DnaK protein. The *dnaK*332 mutation is a point mutation located in the amino-terminal, ATPase domain of DnaK, which could potentially result in an altered affinity of the protein for ATP (8, 2, 1). However, DnaK332 was purified using the standard procedure elaborated for wild-type DnaK (see Materials and Methods) based on the affinity of the DnaK protein for an ATP agarose column, indicating that it retains the ability to bind to ATP.

DnaK332 protein was found to be fully active in the *in vitro* λ DNA replication system based on crude *E. coli* fraction II extracts (Fig. 4.1). As can be seen in Fig. 4.1A, wild-type DnaK alone is unable to replicate the λ DNA when the fraction II extract is derived from the *dnaK*103 *grpE*::cam^r strain. This is due to the lack of GrpE in the system. When GrpE is added to the system, DnaK is able to replicate the DNA efficiently (Fig. 4.1A).

In contrast, DnaK332 is able to replicate λ DNA using fraction II extract derived from *dnaK*103 *grpE*::cam^r without the addition of any GrpE protein, and does so nearly as

Figure 4.1. *In vitro* λdv DNA replication by DnaK and DnaK332. The standard replication assay premixture (15) using fraction II extracts derived from *dnaK*103 *grpE::cam*^{r} (A) incubated with varying concentrations of DnaK, with or without the addition of 1.8μM, GrpE or varying concentrations of DnaK332 without addition of any GrpE; (B) incubated with varying concentrations of GrpE and 1.14μM DnaK or 3μM DnaK332.


A
DnaK+GrpE
DnaK332
DnaK
DNA synthesis (pmol)
DnaK332 or DnaK protein (μM)
0
25
50
75
100
125
150
175
200
0
2
4
6
8
10
12


B
DnaK
DnaK332
GrpE protein (μM)
0
50
100
150
200
0
1
2
3
4

well as wild-type DnaK which is suplemented with GrpE (Fig. 1A). Interestingly, the DnaK332 protein is unable to replicate the λ DNA when *dnaK*103 *grpE*$^{+}$ fraction II extracts are used, though wild-type DnaK is able to do so (results not shown), supporting the idea that GrpE inhibits the ability of the DnaK332 protein to function efficiently. Titration of GrpE protein into this system using fraction II extracts prepared from *dnaK*103 *grpE::cam*^{r} shows that while small concentrations of GrpE slightly stimulate the DnaK332-dependent λ DNA replication activity, larger concentrations strongly inhibit it (Fig. 4.1B). These results strongly support the previously obtained genetic evidence that the DnaK332 protein not only overcomes the need for GrpE, but that the presence of GrpE inhibits its activity (8).

Interaction of DnaK332 with GrpE

Because the DnaK332 protein allows *E. coli* growth and λ DNA replication *in vitro* in the absence of GrpE, and because such activities are inhibited by GrpE's presence, the ability of DnaK332 and wild-type DnaK to interact with GrpE *in vitro* was tested using size exclusion chromatography on an SEC 4000 HPLC column (Fig. 4.2).

As reported in Chapter 3, and as can be seen in Fig. 4.2, DnaK alone behaves on the HPLC column as if it were a globular protein with a molecular mass which is greater

Figure 4.2. Interaction of DnaK and DnaK332 with GrpE in the presence and absence of ATP. (A) Interaction of DnaK with GrpE. ***DnaK + ATP:*** DnaK (20μM) was preincubated for 30 min at 42°C with 200μM ATP in a 40μl reaction mixture containing 25mM Hepes/KOH pH 7.2, 150mM KCl, 25mM NaCl and 5mM $MgCl_2$ and injected onto the SEC 4000 HPLC size exclusion column. The column had been equilibrated with the same buffer as above, but without the addition of ATP. ***GrpE + ATP:*** GrpE protein (20μM) was preincubated with ATP under the same conditions described above. ***DnaK + GrpE:*** DnaK (20μM) was mixed with GrpE (20μM) and incubated for 10 min at 42°C before being injected onto the column (no ATP was in the premixture or the column buffer). ***(DnaK + GrpE)ATP:*** The same reaction was repeated with the difference that 200μM ATP was added to both the premixture and the column buffer. In parallel with these experiments, molecular weight standards were injected onto the column under the same chromatographic conditions: 1. blue dextran (Vo of the column), 2. apoferitine (443,000) 3. β amylase (200,000) 4. BSA (68,000).

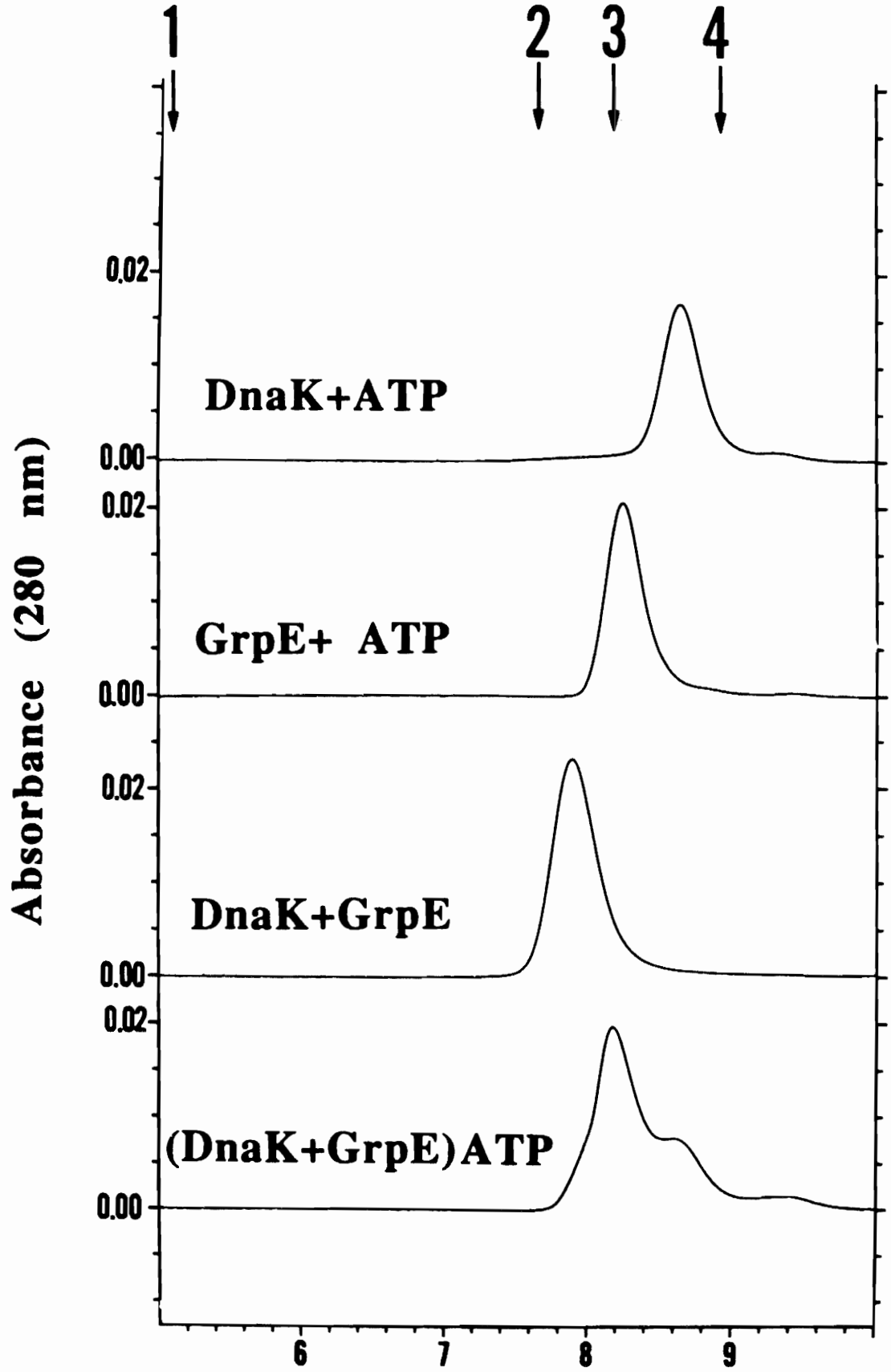
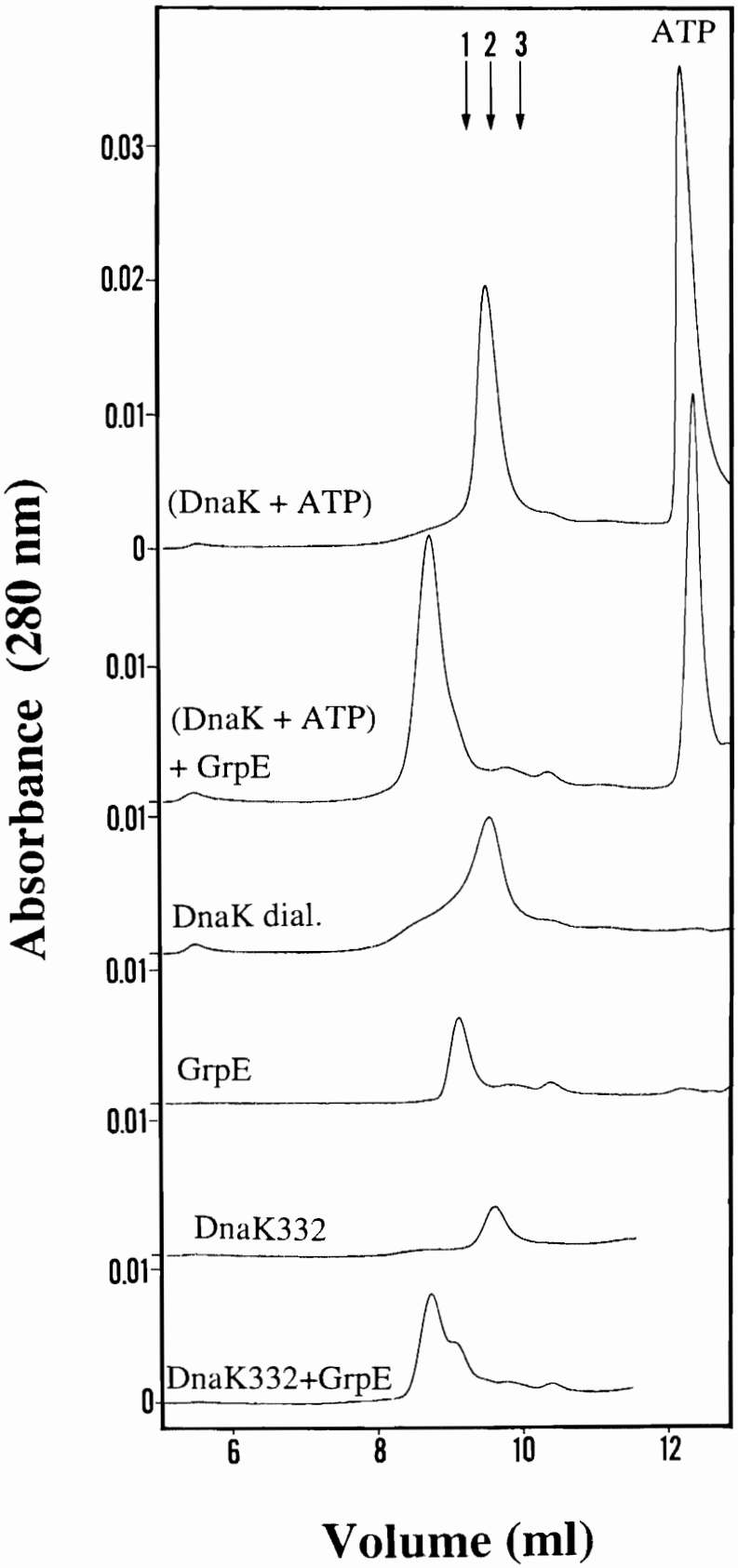

1
2
3
4
0.02
0.00
DnaK+ATP
0.02
0.00
GrpE+ ATP
0.02
0.00
DnaK+GrpE
0.02
0.00
(DnaK+GrpE)ATP
Absorbance (280 nm)
6
7
8
9

Figure 4.2B. Interaction of DnaK332 with GrpE. The size exclusion chromatography was performed as described in (Fig. 4.2 A) above except that a guard column was applied before the SEC 4000 HPLC column. ***DnaK:*** 14μM DnaK after extensive dialysis in the presence of EDTA. ***DnaK + ATP:*** Dialyzed DnaK was preincubated with 140μM ATP for 30 min at 42°C and injected onto the column (no ATP was present in the column buffer). The position of free ATP is also indicated in the chromatograph. ***ATP + DnaK + GrpE:*** 14μM DnaK was first preincubated with 140μM ATP for 30 min at 42°C, after which 18μM GrpE was added, and the mixture was injected onto the column (no ATP was present in the column buffer). ***DnaK332:*** 4μM DnaK332 was chromatographed as described in the legend to Fig. 3. ***DnaK332 + GrpE:*** 4μM DnaK332 was preincubated with 10μM GrpE for 10 min at 42°C and then injected onto the column. In parallel with these experiments, molecular weight standards were applied to the column under the same conditions: 1. β amylase (200,000) 2. Phosphorylase B (94,000) and 3. BSA (68,000).


1 2 3
ATP
0.03
0.02
0.01
0
(DnaK + ATP)
(DnaK + ATP)
+ GrpE
DnaK dial.
GrpE
DnaK332
DnaK332+GrpE
Absorbance (280 nm)
6
8
10
12
Volume (ml)

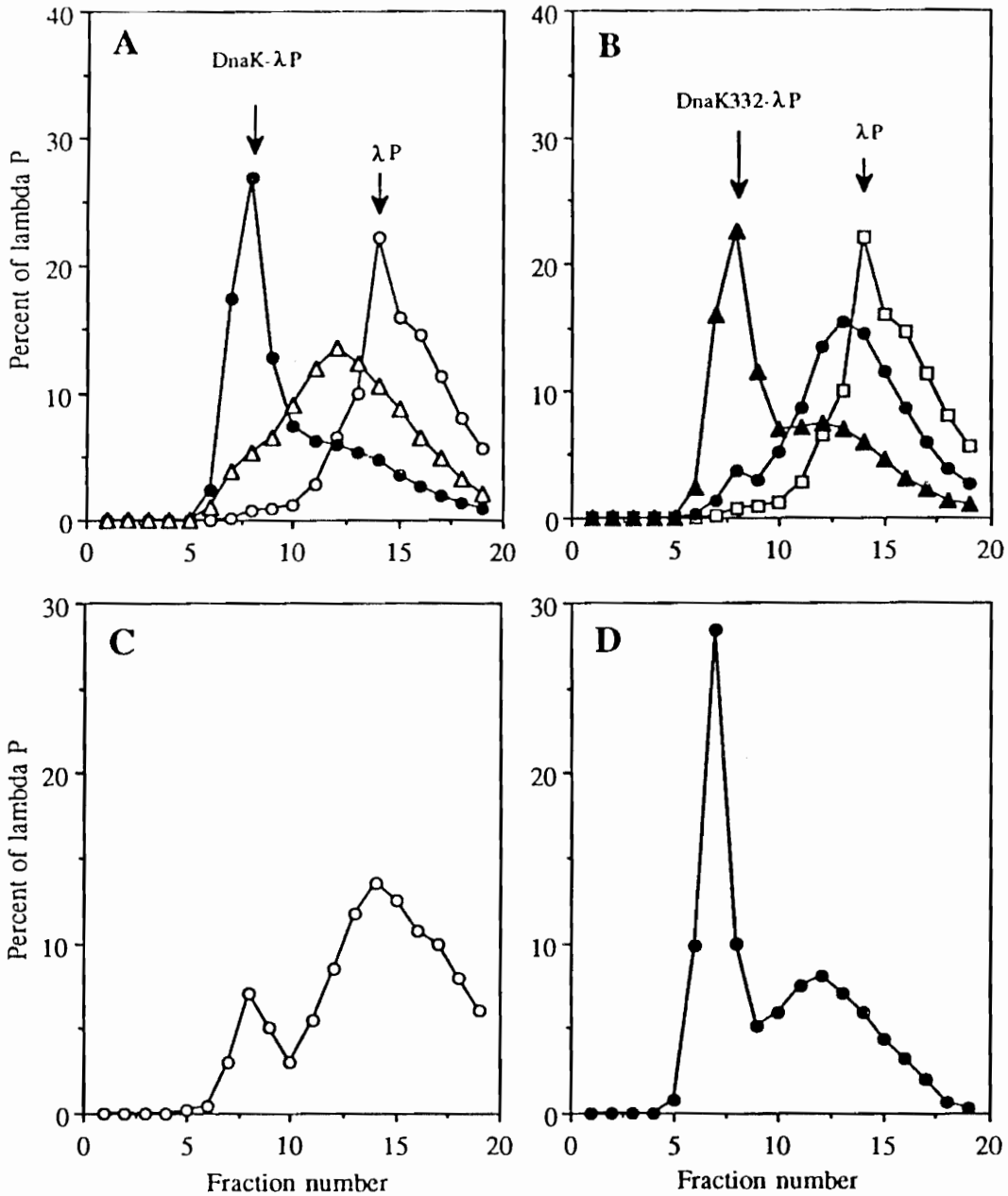
than the monomeric, but less than the dimeric mass of DnaK (Fig. 4.2). The GrpE protein alone behaves on the HPLC column as a tetramer (Fig 4.2A and B). When both DnaK and GrpE are mixed together in equal molar ratios, a GrpE-DnaK complex can be detected by HPLC chromatography (Fig. 4.2). Dissociation of this complex is observed when ATP is added to both the premixture and the column buffer (Fig. 4.2).

The mutant protein DnaK332 behaves on the HPLC size chromatograpy just as the wild-type DnaK protein does (Fig. 4.2B), forming a complex with GrpE in a manner similar to wild-type DnaK (Fig. 4.2B). Also, as with the wild-type DnaK protein, the presence of ATP in the reaction and column buffers results in dissociation of the DnaK332-GrpE complex (results not shown).

Affinity of DnaK332 for λP

In order to monitor the ability of DnaK332 and wild-type DnaK to bind to the λP replication protein, a size exclusion chromatographic assay was performed on a P150 (BioRad) column. As has previously been reported, when [^{14}C]-λP is incubated alone for 15 min at 37°C and chromatographed through the P150 column, it behaves as a monomer or dimer, eluting as a single peak in the 14th fraction (6; Fig. 4.3). The presence of increasing amounts of DnaK in the pre-incubation reaction alters this elution profile such that most of the [^{14}C]-λP elutes with DnaK in

Figure 4.3. Interaction of DnaK and DnaK332 with λP. Incubation of 0.75μM [^{14}C]-λP in the presence or absence of 1.71μM DnaK (A) or 3μM DnaK332 (B). Open triangles in panel A and closed circles in panel B represent the elution profile of [^{14}C]-λP when incubated in the presence of 1mM ATP and DnaK (A) or DnaK332 (B). (C) Elution profile of 0.75μM [^{14}C]-λP when pre-incubated in the presence of DnaK and 2.5μM GrpE. (D) Elution profile of 0.75μM [^{14}C]-λP when pre-incubated in the presence of DnaK332 and 2.5μM GrpE.


A
DnaK-λP
λP
B
DnaK332-λP
λP
C
D
Percent of lambda P
Fraction number
0
10
20
30
40
0
5
10
15
20

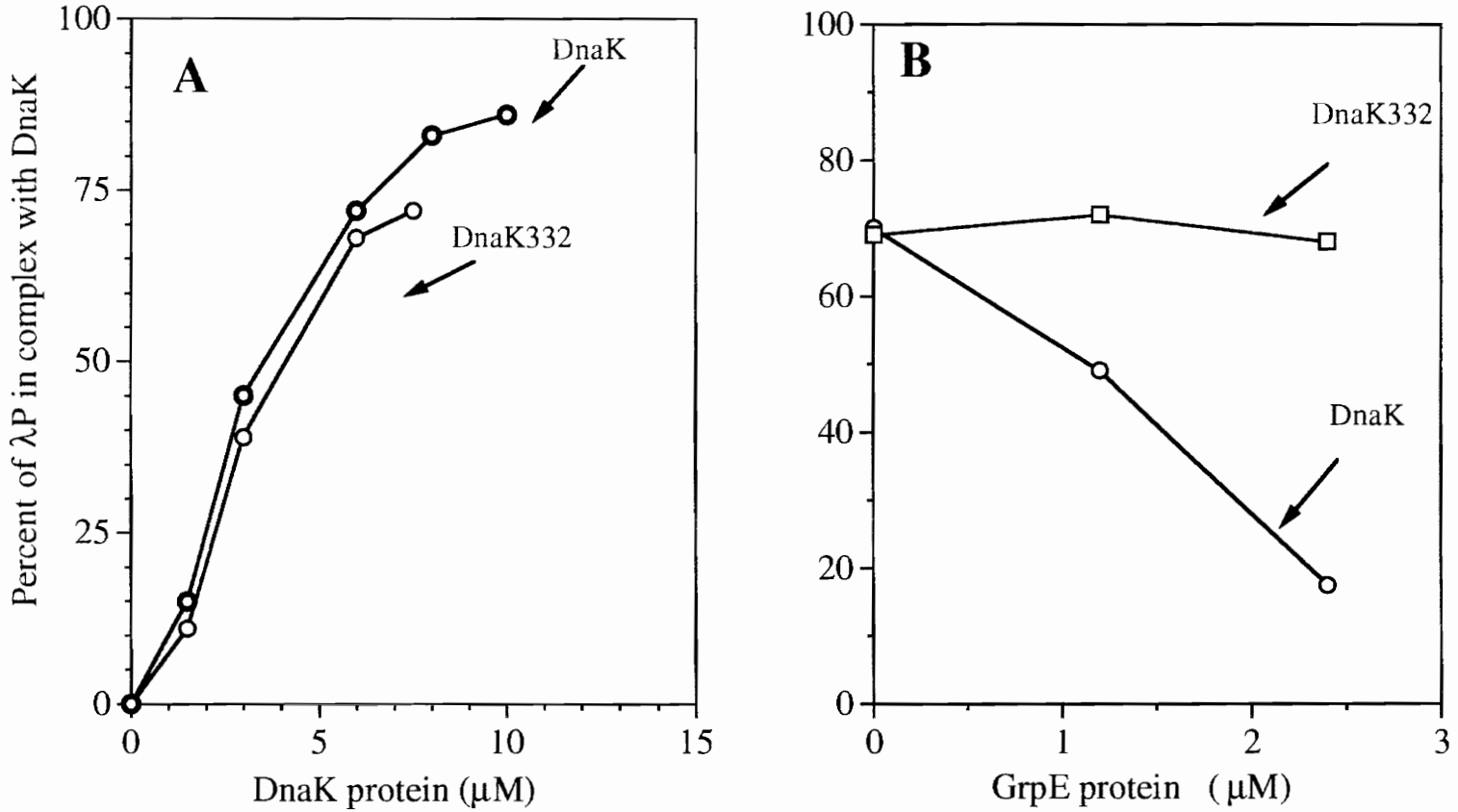
the void volume of the column (Figs. 4.3A and 4.4A). Also as previously demonstrated (6), the presence of ATP partially disrupts the formation of the λP-DnaK complex (Fig. 4.3A). When GrpE is incubated with DnaK and [^{14}C]-λP in the premixture, less [^{14}C]-λP is present in the DnaK complex (Fig. 4.3C).

DnaK332 shows the same affinity for λP as does wild-type DnaK (Figs. 4.3B and 4.4A). As with wild-type, the presence of ATP partially disrupts the DnaK332-λP complex (Fig. 4.3B). Surprisingly, DnaK332 is unable to release λP in the presence of GrpE (Figs. 4.3D and 4.4B), suggesting that DnaK332, which is able to interact with GrpE, can efficiently sequester λP, but cannot be recycled in this reaction when GrpE is present.

Affinity of DnaK332 for Nucleotides
in the Presence and Absence of GrpE

In order to monitor the form of nucleotide bound to DnaK, [α-^{32}P]-ATP was preincubated with DnaK in order to allow a complex to form, and the nucleotide-protein complex was purified from the free nucleotides as previously described (5). This complex was then incubated in the presence or absence of GrpE, and again the protein was separated from the free nucleotide using a small P-60 column. Each fraction from the second column was thin

Figure 4.4. Affinity of DnaK or DnaK332 for λP, and effect of GrpE on the affinity. (A) Incubation of 0.75μM [^{14}C]-λP in the presence of increasing concentrations of DnaK or DnaK332, and calculation of percent of [^{14}C]-λP which elutes with the DnaK or DnaK332. (B) Incubation of 0.75μM [^{14}C]-λP with 1.71μM DnaK or 3μM DnaK332 and increasing concentration of GrpE, and calculation of the percent of [^{14}C]-λP which elutes with DnaK or DnaK332.


A
B
DnaK
DnaK332
Percent of λP in complex with DnaK
DnaK protein (μM)
GrpE protein (μM)
0
25
50
75
100
0
5
10
15
20
40
60
80
1
2
3

layer chromatographed on cellulose PEI plates to determine the amount of ATP versus ADP present (Fig. 4.5).

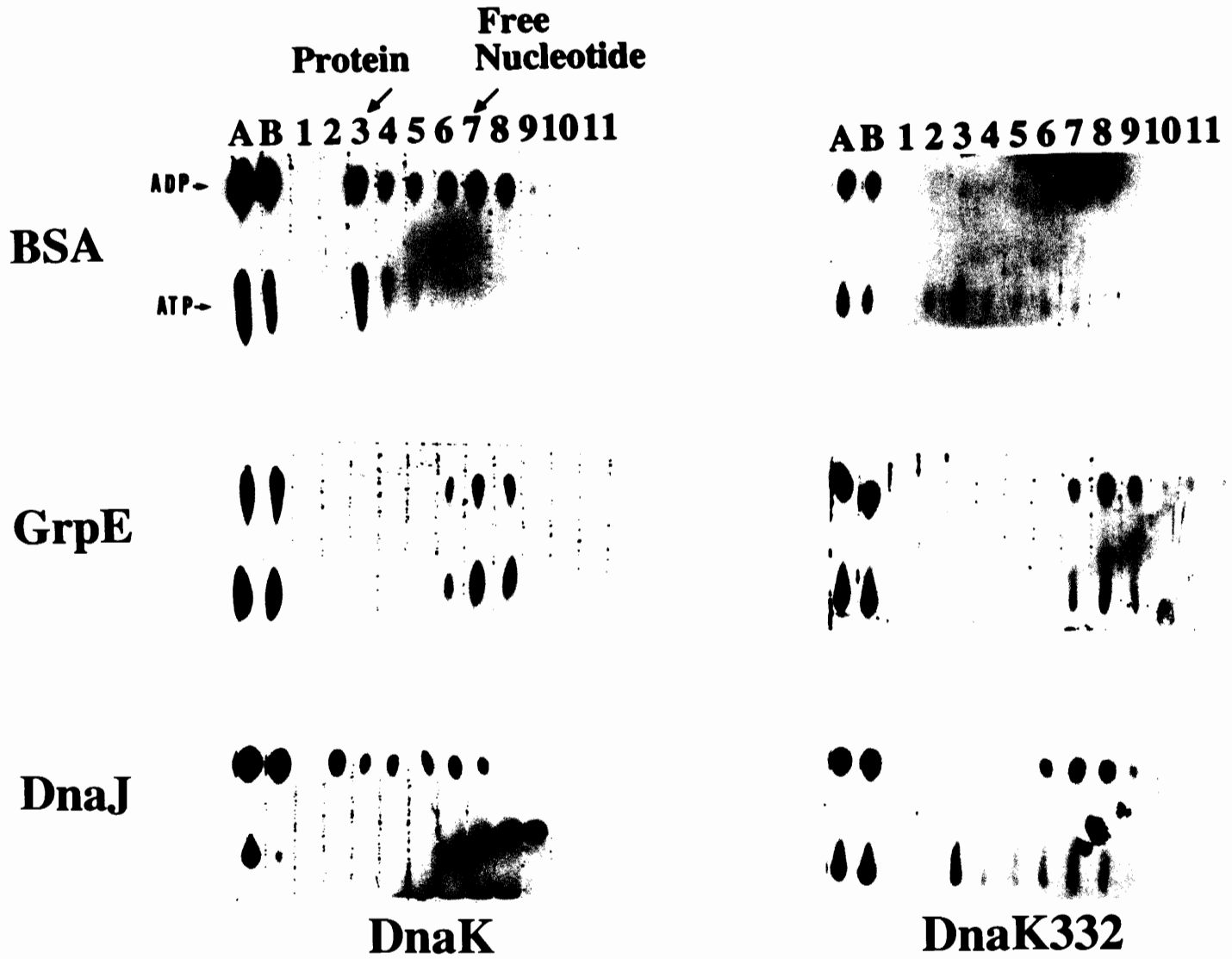
As can be seen in Fig. 4.5, isolated DnaK has nearly equal amounts of ATP and ADP bound to it. As published previously, GrpE releases both ATP and ADP with equal efficiency from DnaK (5; Fig. 4.5).

Surprisingly, the DnaK332 mutant protein appears to release ADP without the aid of GrpE, although ATP is still bound to it, suggesting that DnaK332 may overcome the need for GrpE by spontaneously releasing ADP upon ATP hydrolysis (Fig. 4.5). GrpE does, however, release the remaining ATP from DnaK332, indicating that, at some level, GrpE can act catalytically upon DnaK332 (Fig. 4.5).

As previously reported (5), the presence of DnaJ greatly stimulates the hydrolysis of the ATP which is bound to DnaK (Fig. 4.5). Surprisingly, DnaJ does not appear to have any effect on DnaK332's rate of ATP hydrolysis, suggesting that possibly, in addition to working independently of GrpE, DnaK332 works independently of, or does not interact with DnaJ (Fig. 4.5).

From the control experiment presented in Fig. 4.5 (incubation of DnaK and DnaK332 with BSA), it appears that DnaK332 has nearly the same ATPase activity as wild-type DnaK protein. Indeed, further substrate saturation experiments indicated that DnaK332's ATPase activity was

Figure 4.5. Effect of GrpE or DnaJ on DnaK- or DnaK332-nucleotide complex. Incubation of 2.2μM DnaK- (column 1) or DnaK332--[α-^{32}P]-nucleotide complex (column 2) for 1 min in the presence of 2.9μM BSA (row 1), 3.1μM DnaJ (row 2) or 2.4μM GrpE (row 3), under conditions previously described (5). A sample of each mixture was applied to PEI cellulose paper immediately following the addition of BSA, DnaJ or GrpE (lanes A), and following a 1 min incubation (lanes B). The remaining mixture was then applied to a P-60 column, and a sample from each 2-drop eluted fraction was applied to the PEI cellulose paper (lanes 1-11) for chromatography, as previously described (5).


Protein
Free Nucleotide
A B 1 2 3 4 5 6 7 8 9 10 11
ADP
ATP
BSA
GrpE
DnaJ
DnaK
A B 1 2 3 4 5 6 7 8 9 10 11
DnaK332

not significantly altered compared to that of wild-type DnaK (Fig. 4.6).

Behavior of DnaK332 in the Presence of DnaJ

It has been reported that the presence of ATP has a profound effect upon the conformation of wild-type DnaK as evidenced by as alteration of the cleavage pattern of the protein upon digestion with trypsin (7). However, in the presence of DnaJ, this effect is not seen. The trypsin cleavage pattern of DnaK in the presence of DnaJ and ATP looks similar to that of DnaK in the absence of ATP, as if DnaJ shields DnaK from ATP (11; Fig. 4.7).

As with wild-type DnaK, DnaK332 exhibits the same alteration in trypsin cleavage pattern in the presence, versus the absence, of ATP (Fig. 4.7). However, in the presence of DnaJ, little if any shielding effect is seen; the cleavage pattern of DnaK332 in the presence of ATP and DnaJ is similar to that of DnaK332 in the presence of ATP alone (Fig. 4.7). This result further indicates that DnaK332 may not interact productively with DnaJ.

Discussion

It is generally perceived that the DnaK, DnaJ and GrpE proteins of *E. coli* interact as a "chaperone machine." Within this machine, DnaK is the "work-horse," responsible

Figure 4.6. ATPase activity of DnaK or DnaK332. A reaction mixture of 1.7μM DnaK or DnaK332 in the presence of increasing concentrations of ATP. Reaction conditions are as previously described (5). Vmax of DnaK = 25 nmoles/min/mg, Km = 0.22mM. Vmax of DnaK332 = 30 nmoles/min/mg, Km = 0.20mM.

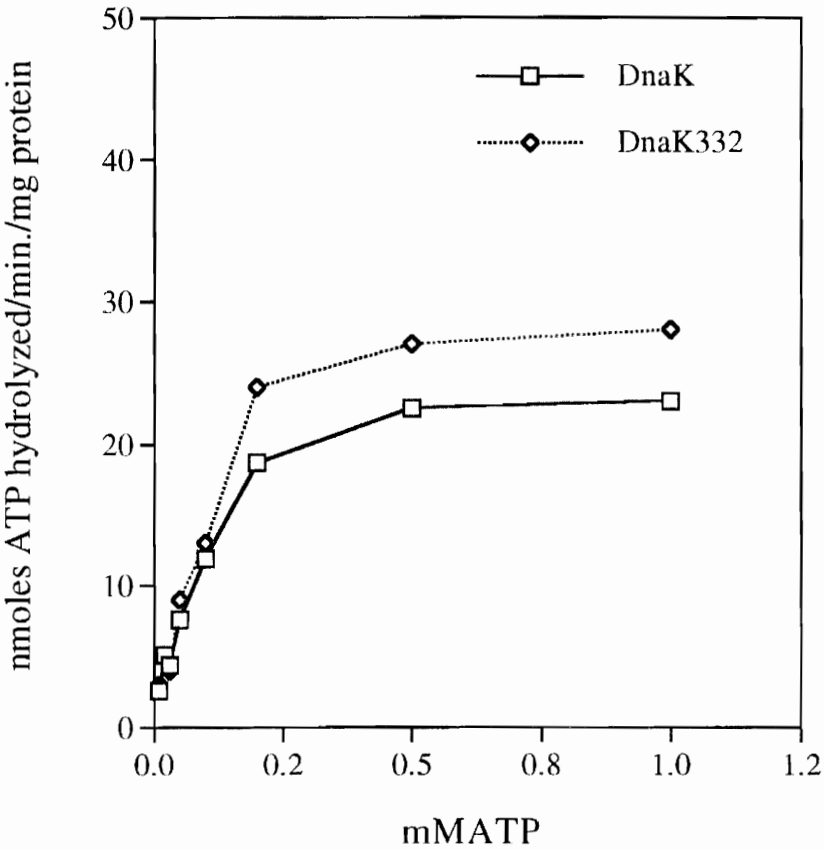
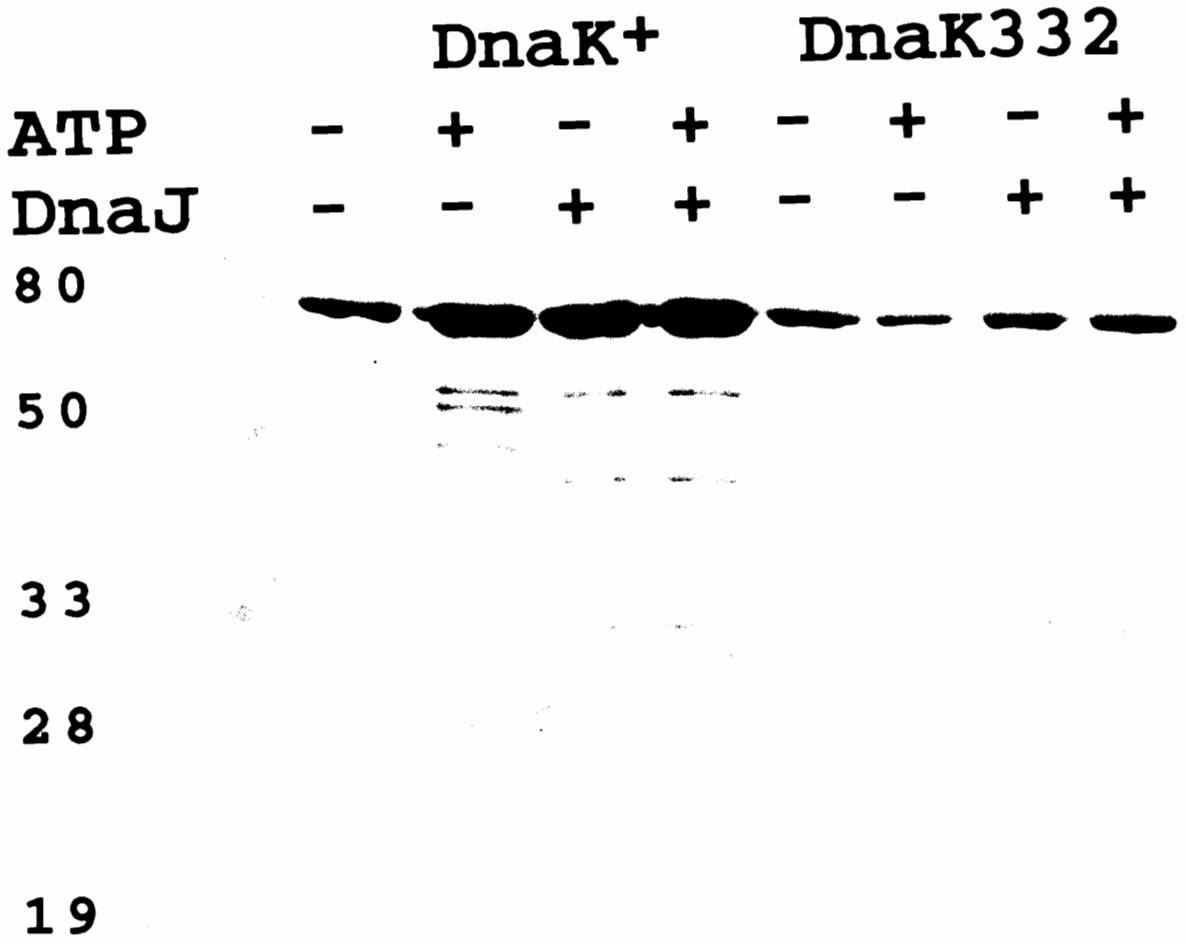

50
40
30
20
10
0
0.0
0.2
0.5
0.8
1.0
1.2
DnaK
DnaK332
nmoles ATP hydrolyzed/min./mg protein
mMATP

Figure 4.7. Trypsin digestion pattern of DnaK or DnaK332 in the presence or absence of ATP and DnaJ. 2.28μM DnaK (lanes 1 - 4) or DnaK332 (lanes 5 - 8) digested with 0.12μg Trypsin for 25 min at room temperature alone (lanes 1 and 4), or in the presence of 2.5mM ATP (lanes 2 and 6); 3.9μM DnaJ (lanes 3 and 7); or 3.9μM DnaJ and 2.5mM ATP (lanes 4 and 8). Molecular weight standards are as indicated in the figure (kDa).


DnaK+
DnaK332
ATP
DnaJ
80
50
33
28
19

for the actual chaperoning activities, and DnaJ and GrpE are DnaK's cohorts, responsible for "seeing" that DnaK is "ready" to function. In this regard, two specific roles have previously been suggested for GrpE: (a) helping DnaK to be recycled from its complex with substrates (5, 9), and (b) helping DnaK to better recognize its proper substrates (9, 12). In addition, it has been demonstrated that GrpE releases ATP and ADP from complex with DnaK *in vitro* (5). In this chapter, based on the findings regarding (a) the relative affinities of the DnaK vs. DnaK332 proteins for nucleotide, and (b) the natures of their interactions with GrpE, this *in vitro* reaction has been shown to have biological significance, and additional details for the model of GrpE's interaction with DnaK can be provided.

The single point mutant DnaK332, which substitutes the positively charged Arg with the nonpolar Leu, is able to bypass the need for GrpE both *in vivo* (8) and *in vitro*. In the presence of DnaK332, *in vitro* λ DNA replication does not require the presence of GrpE, and is, in fact, inhibited by its presence at concentrations known to support replication by wild-type DnaK. It was previously shown that in the multistep initiation reaction of λ DNA replication, DnaK first binds to λP which is in the preprimosomal complex. Next, in an ATP-dependent reaction, DnaK either releases λP from the preprimosomal complex (the GrpE-independent reaction) or translocates it in such a way

that λP is no longer in a complex with DnaB helicase (the GrpE-dependent reaction; 14, 13).

From the work presented in this chapter, it is clear that both DnaK and DnaK332 bind to λP equally well, but that, surprisingly, in contrast to the wild-type situation, the presence of GrpE will not allow the release of λP from its complex with DnaK332. One could imagine a situation such that, in the wild-type case, GrpE competes with λP for binding to DnaK, whereas in the case of DnaK332, such competition does not occur, for example, because DnaK332 does not interact with GrpE. However, this cannot be the case since, from the HPLC experiments it is clear that DnaK332 interacts with GrpE as well as wild-type DnaK does. Furthermore, the work presented in Chapter 3 demonstrated that truncation of 94 amino acids from the carboxyl-terminal end of DnaK, which seriously alters the ability of the protein to form a stable complex with λP, does not interfere with the binding of DnaKc94 to GrpE. Not only do these findings preclude the possibility of a competition for binding to DnaK between λP and GrpE, they also suggest that GrpE interacts with DnaK through the amino-terminal region, rather than through the carboxyl-terminal region. This further suggests that GrpE must change the conformation of the carboxyl-terminal domain through its actions at the amino-terminus in order that it might affect DnaK's interaction with substrate. It seems possible,

then, that in the case of DnaK332, such a conformational change may not occur upon binding to GrpE. This, in turn, would lead to a block in the release of substrates such as λP.

Previously, it was established that DnaK undergoes dramatic conformational changes in the presence of ATP which were thought to result in the release of λP from its complex with DnaK (7). GrpE could indirectly modulate binding and release of λP from DnaK by modulating binding and release of ATP or ADP from DnaK. This chapter clearly provides evidence that the mutant DnaK332 protein does not require GrpE to release ADP. In this case, ADP is released spontaneously, while wild-type DnaK requires GrpE to release ADP.

One interpretation of this result is that GrpE-dependent release of ADP from DnaK is required for the release of substrate from DnaK. GrpE is not required in the case of DnaK332 because this protein spontaneously releases ADP. From this point of view, it would be very interesting to see if any of the eukaryotic DnaK analogs also release ADP spontaneously, since no eukaryotic GrpE analog has been discovered.

In both the *in vivo* and *in vitro* λ DNA replication systems ATP and ADP are always present. Assuming that DnaK332 spontaneously releases ADP (but not ATP), the equalibrium of the reaction will be shifted to the DnaK332-

ATP form. In the case of the wild-type protein, the same balance between DnaK-ATP and DnaK-ADP can be accomplished by the presence of GrpE. Thus GrpE could be viewed as a trigger factor which maintains a proper balance between the active and nonactive forms of DnaK. Whether the DnaK-ATP or DnaK-ADP form is the active form in λ DNA replication is still unclear. Furthermore, there is a possibility that the active form could depend on the particular substrate (e.g., σ^{32} versus λP).

It has previously been shown that increasing concentrations of GrpE inhibit DnaK-dependent λ DNA replication (14). Here, it is shown that in the case of DnaK332-dependent λ DNA replication, the system is much more sensitive to the presence of GrpE. Even small amounts of GrpE severely inhibit initiation of DnaK332-dependent λ DNA replication. It is possible that in this case, GrpE not only releases ADP, but also ATP from DnaK332 in a manner that inhibits λ DNA replication.

When DnaK is preincubated with ATP, it is possible to isolate almost equal amounts of the DnaK-ATP or DnaK-ADP bound forms. Both of these forms are substrates for GrpE, which releases ATP and ADP from DnaK and then forms a stable complex with DnaK. At the same time, it has been shown that when ATP is present in both the reaction mixture and during chromatography, the DnaK-GrpE complex is dissociated, and DnaK can again form a complex with ATP or

ADP (this chapter and Chapter 3). This result indicates that GrpE is required to disrupt the DnaK-ATP complex, but at the same time, ATP is required to disrupt the DnaK-GrpE complex. In this way, it appears that both DnaK and GrpE are recycled in this reaction. *In vivo*, then, it is likely that at least three forms of DnaK (which are not complexed with substrate) exist at the same time: DnaK-ATP, DnaK-GrpE and DnaK-ADP, and it is possible that the DnaK-GrpE complex is an intermediate between the DnaK-ADP and DnaK-ATP forms.

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CHAPTER 5

PURIFICATION AND PROPERTIES OF THE DnaK325 MUTANT PROTEIN

Abstract

The *dnaK*325 mutation was isolated on the basis of its ability to compensate for the temperature sensitive phenotype of the *grpE*280 mutation. Subsequent genetic analyses indicated that this mutation allowed *grpE*-independent *E. coli* growth and bacteriophage λ replication. Data presented in this chapter demonstrate that the DnaK325 mutant protein does indeed function independently of GrpE, and that GrpE is inhibitory to its ability to function. They further indicate that DnaK325 may achieve GrpE independence in a mechanism similar to that of DnaKc94, through an increased ATPase activity and an alteration in its conformation.

Introduction

The *dnaK*325 mutation was isolated using the same selection as the *dnaK*332 mutation, based on its ability to allow the growth of *grpE*280 at 43°C (5; Chapter 2). Like *dnaK*332, the *dnaK*325 mutation is a single base change. This change results in the substitution of Ser for the conserved Gly at position 10 in the amino acid sequence of the ATPase domain of DnaK (5; Chapter 2). As with the *dnaK*332 *grpE*280 double mutant, *dnaK*325 *grpE*280 mutant cells were found to support the growth of wild-type bacteriophage λ, and to be unable to replicate the λ*grpE*$^+$ transducing bacteriophage (5; Chapter 2). Similarly, the *dnaK*325 mutation was found

to allow a deletion of the *grpE* gene in *E. coli*. However, *dnaK*325 appears to differ from *dnaK*332 in this respect in that while *dnaK*332 cells allow deletion of *grpE* only up to 33°C, *dnaK*325 allows such a deletion up to 43°C. Furthermore, *dnaK*325 *grpE*::Ωcam^r cells are able to support the growth of bacteriophage λ in the complete absence of any *grpE*, while *dnaK*332 *grpE*::Ωcam^r cells are unable to do so (5; Chapter 2). Such results indicate that, like DnaK332, DnaK325 is poisoned by the presence of GrpE, but that unlike DnaK332, DnaK325 functions entirely independent of the need for GrpE. Therefore, it seems likely that DnaK325 overcomes the need for GrpE in a manner different from that of DnaK332.

GrpE has been shown biochemically to influence the ATPase activity of DnaK (3), the conformation of DnaK (6; Chapter 3), and the ability of DnaK to interact with substrate (7, 9, 4). As enumerated in Chapters 1 and 3, GrpE causes bound ATP or ADP to be released from DnaK, and in concert with DnaJ's stimulation of the hydrolysis of ATP to ADP, stimulates the ATPase activity of DnaK by approximately 50-fold (3). GrpE has also been found to cause a change in the conformation of DnaK in the presence of ATP which is detectable by changes in the fluorescence of the tryptophan located in DnaK's ATP-binding domain (6; Chapter 3). GrpE has been found to cause the release of λP from DnaK (7), and is also thought to help DnaK to

"identify" its proper substrates (7, 9). Since DnaK325 is able to completely bypass the need for GrpE, it should have altered biochemical properties in one or more of the above functions, if the effect of GrpE on these functions has biological relevance.

This chapter deals with the purification and biochemical characterization of the DnaK325 mutant protein as regards its ability to replicate λ DNA in the presence and absence of GrpE, its affinity for nucleotides in the presence and absence of GrpE, its ATPase activity, its affinity for substrates, and its conformation in the presence and absence of ATP. The results of this characterization indicate that the DnaK325 protein may overcome the need for GrpE, as the DnaKc94 mutant protein does, through an increase in its ATPase activity and an alteration of conformation.

Materials and Methods

Proteins

Proteins of 90% or greater purity were used. GrpE protein was purified as described in Chapter 3.

DnaJ protein was purified according to (10) and (13), and was the kind gift of Dr. Daniel Wall.

The purifications of wild-type DnaK and DnaK325 were done as previously described (12), with the modifications described in Chapter 3. DnaK325 protein was isolated from

E. coli cells containing a single copy of *dnaK*325 on the bacterial chromosome which were grown and lysed as described in Zylicz and Georgopoulos, 1984 (11).

λP protein was purified according to the method outlined in (8). ^{14}C-labeled λP was purified according to (2).

λ DNA Replication Assay

The *in vitro* λ DNA replication assays were performed as previously described (12).

Size Chromatography

Size chromatography on a P-60 gel filtration column was performed as described in (3). Size chromatography on P-150 was performed as previously described (4) with the minor modifications as described in the text and figure legends.

ATPase Assay

ATPase assays were done as previously described (3).

Trypsin Digestion Assay

Trypsin digestion assays were done as previously described (1).

Results

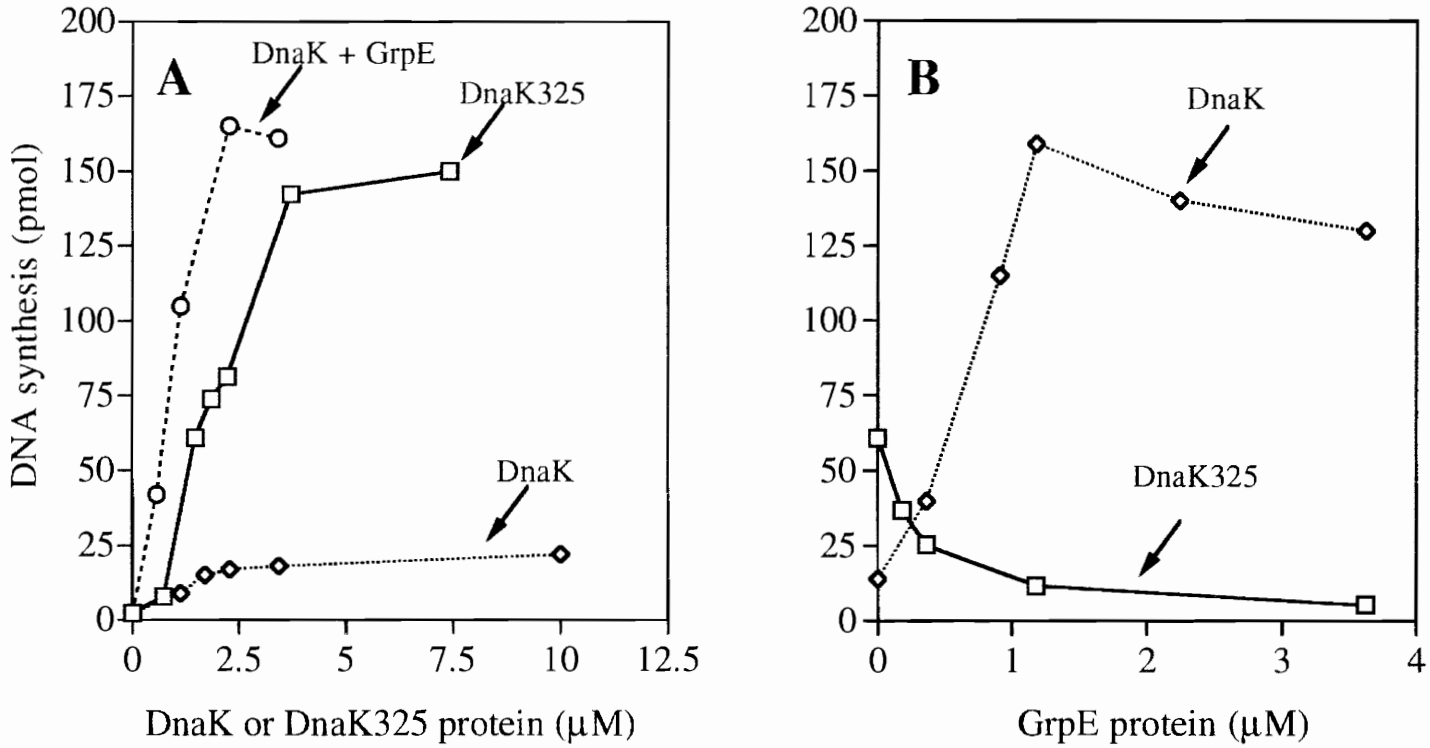
λ DNA Replication Activity

The DnaK325 protein was purified using the standard procedures for DnaK, as described in Chapter 3. Although the DnaK325 mutation resides in the ATPase domain of the protein (5), the protein was purified based on its affinity for an ATP agarose column, indicating that the mutation does not result in a significantly decreased affinity of the protein for ATP.

Using the standard λDNA replication assay based on crude *E. coli* fraction II extracts, the DnaK325 mutant protein was found to be fully active in λ DNA replication (Fig 5.1). This assay was performed using fraction II extracts from the *dnaK*103 *grpE*::Ωcam^r mutant strain. As can be seen in Fig. 5.1, wild-type DnaK protein is unable to replicate the λ DNA efficiently in this system in the absence of GrpE. However, the DnaK325 protein is able to do so, and does so nearly as well as the wild-type DnaK protein which is supplimented with GrpE.

As expected from previously obtained genetic results, while the addition of increasing concentrations of GrpE to the system has a stimulatory effect on DnaK's ability to replicate the DNA, GrpE has a dramatic inhibitory effect on DnaK325's ability to replicate λ DNA. These results support genetic conclusions that DnaK325 is able to

Figure 5.1. *In vitro* λdv DNA replication by DnaK and DnaK325. The standard replication assay premixture (12) using fraction II extracts from *dnaK*103 *grpE::cam*^{r} bacteria (A) incubated with varying concentrations of DnaK, with and without addition of 1.8μM, GrpE or varying concentrations of DnaK325 in the absence of GrpE; (B) incubated with varying concentrations of GrpE and 1.14μM DnaK or 3μM DnaK325.


A
DnaK + GrpE
DnaK325
DnaK
DNA synthesis (pmol)
200
175
150
125
100
75
50
25
0
0
2.5
5
7.5
10
12.5
DnaK or DnaK325 protein (μM)
B
DnaK
DnaK325
200
175
150
125
100
75
50
25
0
0
1
2
3
4
GrpE protein (μM)

function very efficiently in the absence of GrpE, and is poisoned by its presence (5).

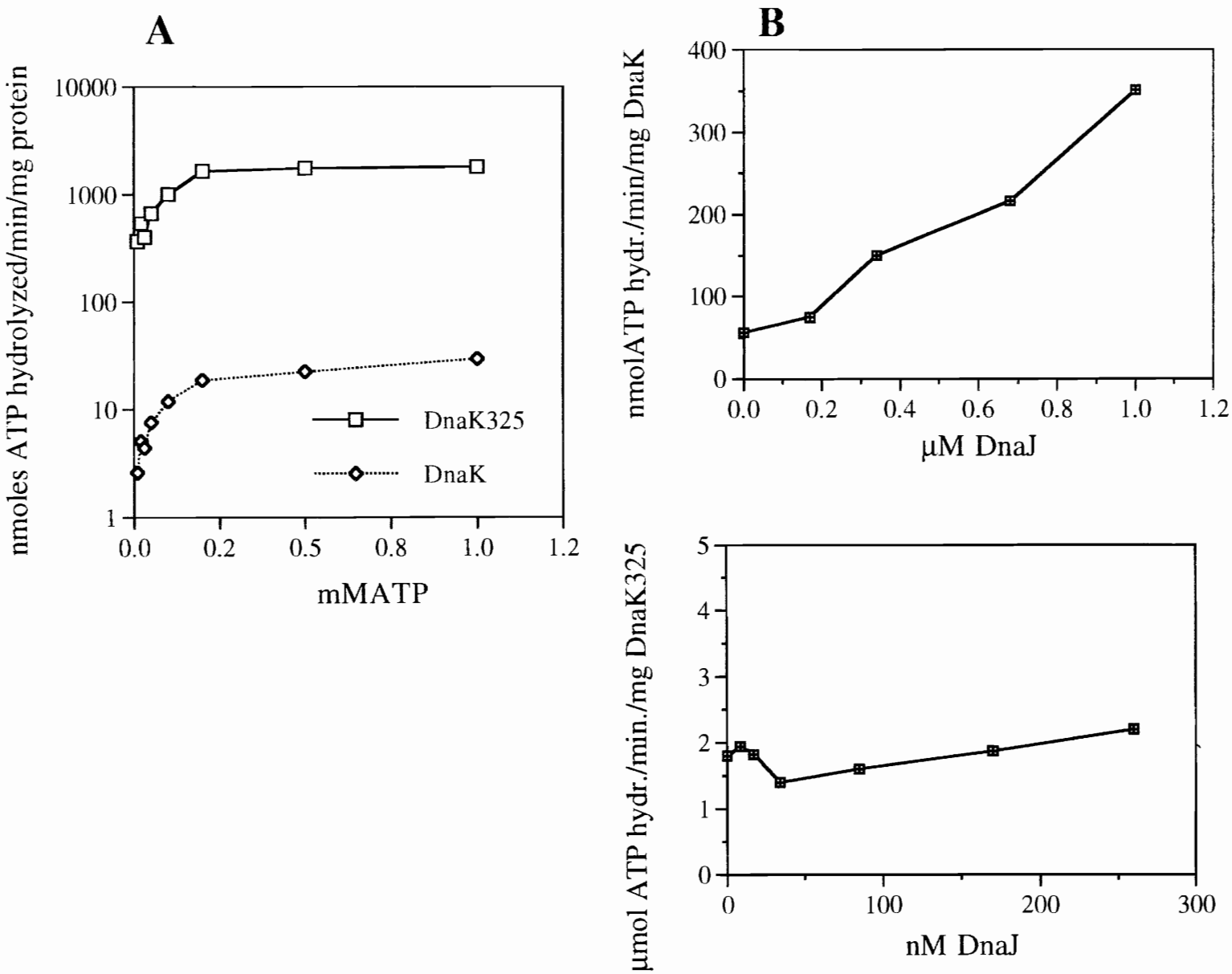
Affinity of DnaK325 for λP

In order to examine the ability of DnaK325 to interact with one of DnaK's physiologic substrates the λP protein, a size exclusion chromotography assay was performed using a P150 (BioRad) column. The results presented in Fig. 5.2 indicate that while DnaK325 is able to bind to the λP protein, it has an approximately 2-fold reduced affinity for the protein as compared with wild-type DnaK. As previously reported, the presence of either ATP or GrpE has the effect of releasing λP from DnaK (4, 7). Similarly, DnaK325 releases λP in the presence of either ATP (results not shown) or GrpE (Fig. 5.2B).

Affinity of DnaK325 for Nucleotides
in the Presence and Absence of GrpE

Because the *dnaK*325 mutation resides in the ATPase domain of the protein, and because GrpE is known to release nucleotide from DnaK, it seems possible that DnaK325 could have an altered affinity for nucleotide, or altered ATPase activity. In order to determine if this were the case, [α-^{32}P]-ATP was pre-incubated with DnaK or DnaK325 to allow complex formation, and the nucleotide-protein complex was separated from free nucleotides as previously described

Figure 5.2. Affinity of DnaK325 for λP. (A) Incubation of 0.75μM [^{14}C]-λP in the presence of increasing concentrations of DnaK or DnaK325. (B) Incubation of 0.75μM [^{14}C]-λP with 1.71μM DnaK or 6.3μM DnaK325 and increasing concentrations of GrpE.


A
nmoles ATP hydrolyzed/min/mg protein
10000
1000
100
10
1
0.0
0.2
0.5
0.8
1.0
1.2
mMATP
DnaK325
DnaK
B
nmolATP hydr./min/mg DnaK
400
300
200
100
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
µM DnaJ
µmol ATP hydr./min./mg DnaK325
5
4
3
2
1
0
0
100
200
300
nM DnaJ

(3). This complex was then incubated in the presence or absence of GrpE or DnaJ, and, again, the protein was separated from the free nucleotide using a small P-60 column. Each fraction from this second column was separated by means of thin layer chromatography on cellulose PEI to determine the amount of ATP vs ADP bound to or released from the DnaK wild-type or mutant proteins (Fig 5.3).

As can be seen in the control experiment shown in Fig 5.3 (DnaK incubated in the presence of BSA), and as published previously, wild-type DnaK protein has nearly equal amounts of ATP as ADP bound to it (3). By contrast, under the same conditions, DnaK325 protein has significantly more ADP than ATP bound to it, indicating that it may have a higher ATPase activity than wild-type DnaK. Indeed, substrate saturation experiments done to determine the ATPase activity of DnaK325 indicate that its Vmax is nearly 80-fold higher than that of DnaK, though the Km remains approximately the same (Fig. 5.4).

As reported previously, the presence of GrpE has the effect of releasing bound nucleotide (both ATP and ADP) from wild-type DnaK (3). As can be seen in Fig. 5.3, this effect extends to DnaK325. GrpE releases bound ATP and ADP from DnaK325.

The presence of DnaJ has been shown to greatly stimulate the hydrolysis of ATP which is bound to DnaK (3).

Figure 5.3. Effect of GrpE and DnaJ on DnaK- or DnaK325-nucleotide complex. Incubation of 2.2μM DnaK- (column 1) or DnaK325-[α-^{32}P]-nucleotide complex (column 2) for 1 min in the presence of 2.9μM BSA (row 1), 3.1μM DnaJ (row 2) or 2.4μM GrpE (row 3), under conditions previously described (3). A sample of each mixture was applied to PEI cellulose paper immediately following the addition of either BSA, DnaJ or GrpE (lanes A), and following the 1 min incubation (lanes B). The remaining mixture was then applied to a P-60 column, and a sample from each 2-drop fraction which was eluted from the column was applied to the PEI cellulose paper (lanes 1-11) for chromatography, as previously described (3).

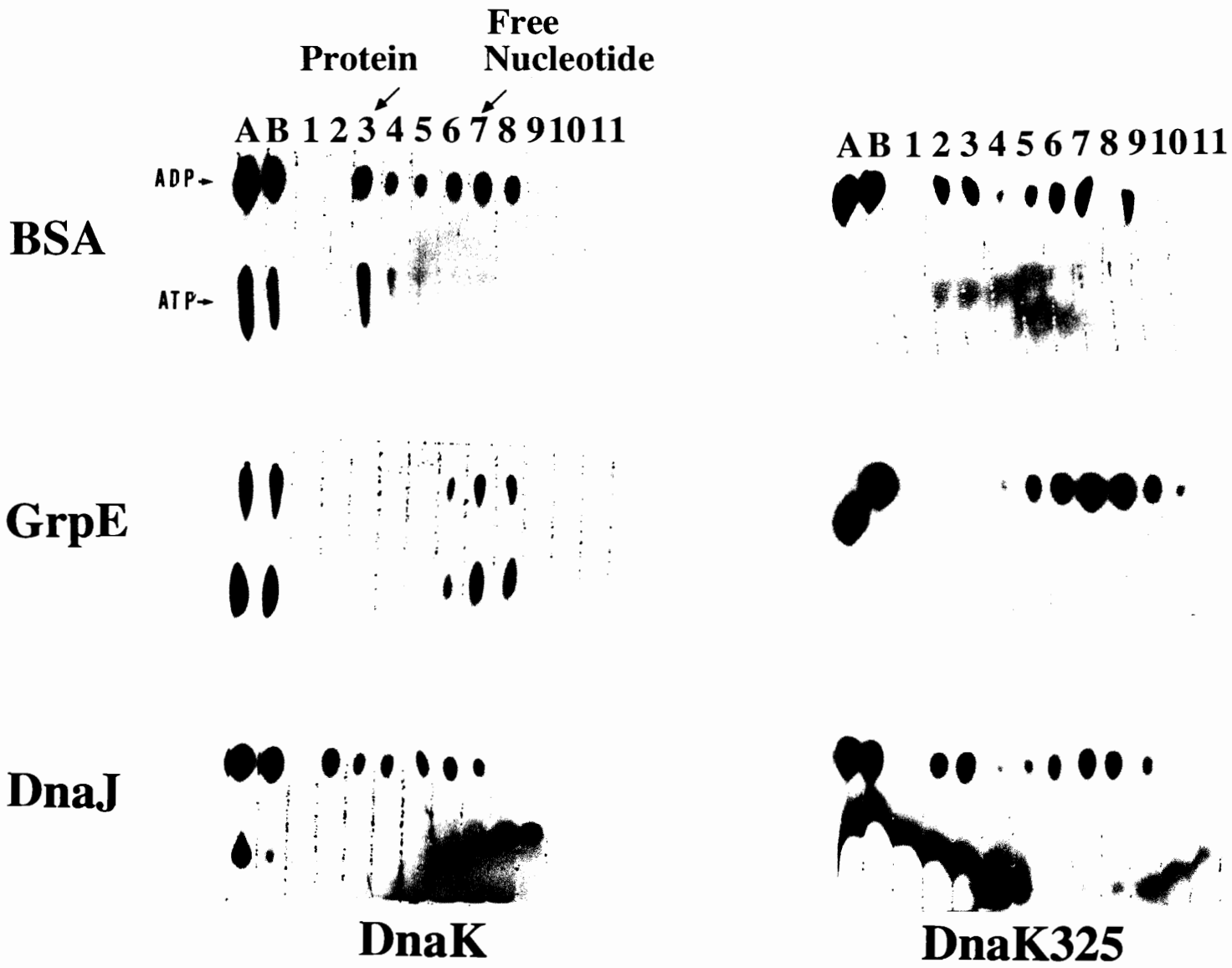
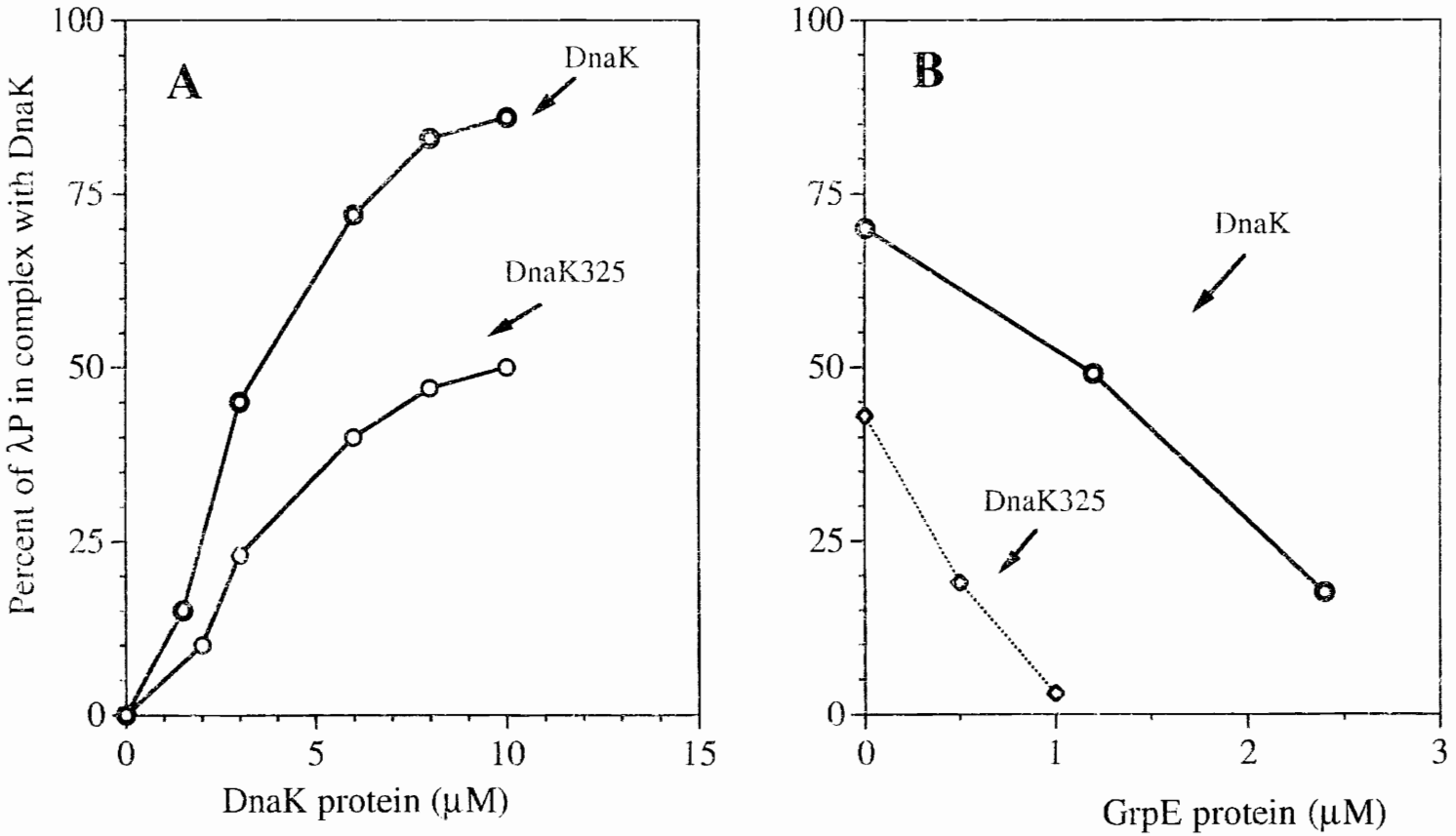

Protein
Free Nucleotide
A B 1 2 3 4 5 6 7 8 9 10 11
ADP
ATP
BSA
GrpE
DnaJ
DnaK
A B 1 2 3 4 5 6 7 8 9 10 11
DnaK325

Figure 5.4. ATPase of DnaK and DnaK325, and effect of DnaJ and GrpE on this activity. (A) A reaction mixture of 1.7μM DnaK or 1.7nM DnaK325 in the presence of increasing concentrations of ATP. Reaction conditions are as previously described (3). Vmax of DnaK = 38 nmoles/min/mg, Km = 0.22mM. Vmax of DnaKc94 = approximately 3000 nmoles/min/mg, Km = 0.21mM. (B) Reaction mixtures of 0.57μM DnaK, 0.83μM GrpE and 1mM ATP or 17nM DnaK325, 34nM GrpE and 1mM ATP with varying concentrations of DnaJ.


A
B
DnaK
DnaK325
Percent of λP in complex with DnaK
DnaK protein (μM)
GrpE protein (μM)
0
25
50
75
100
0
5
10
15
1
2
3

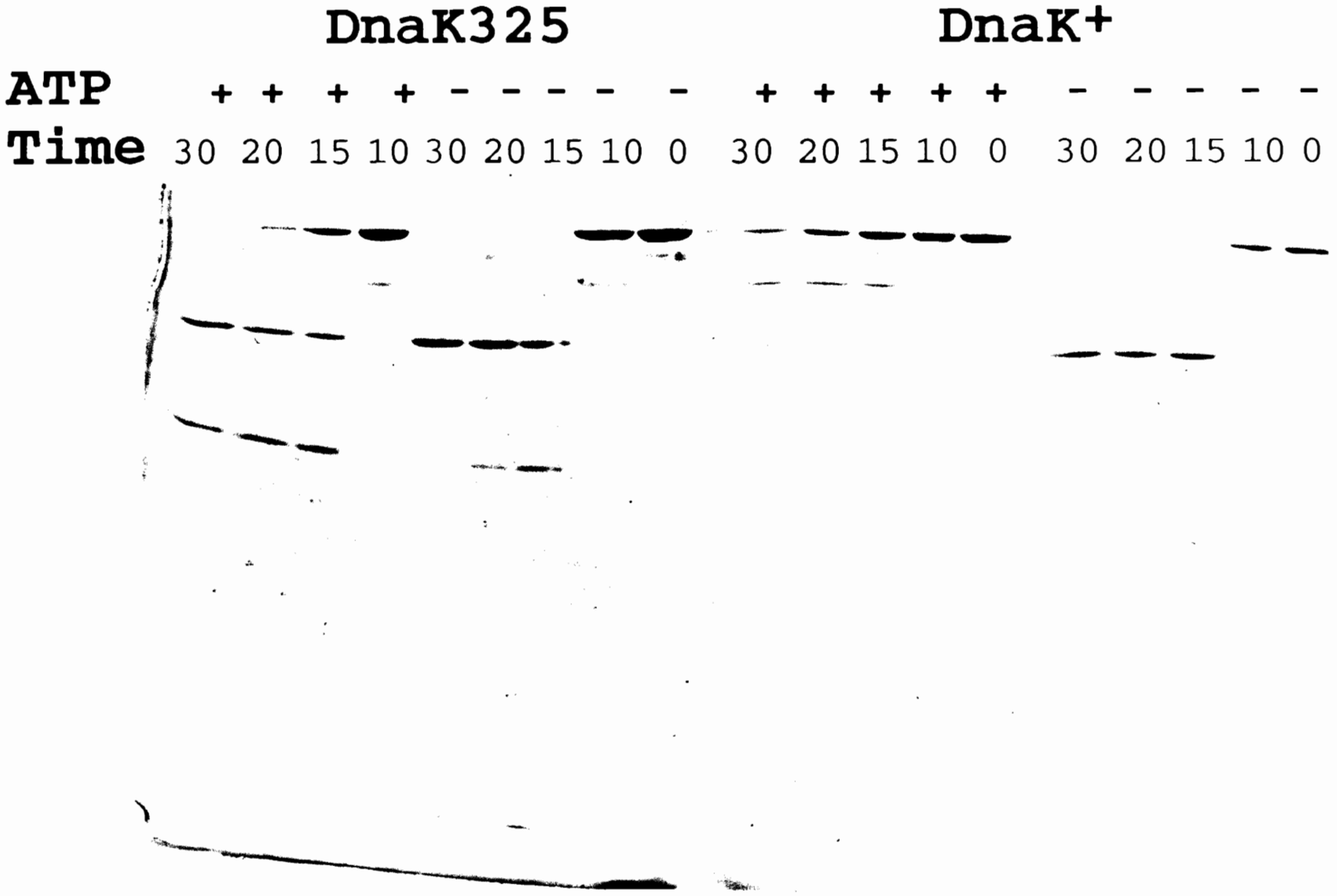
Because DnaK325's rate of ATP hydrolysis is already very high (Figs. 5.3 and 5.4), it is difficult to determine whether the presence of DnaJ has any further stimulatory effect on DnaK325's rate of ATP hydrolysis or not (Fig. 5.3). Furthermore, when the ATPase activity of DnaK325 in complex with GrpE was assayed in the presence of increasing concentrations of DnaJ, it was found that DnaJ did not further stimulate the ATPase activity of this protein, as it does for wild-type DnaK (3), indicating that stimulation of the ATPase activity by DnaJ is not rate limiting for this mutant protein (Fig. 5.4).

Conformation of DnaK325 in the Presence and Absence of ATP

Because GrpE is known to affect the conformation of DnaK in response to ATP, it seems possible that the DnaK325 mutant may overcome the need for GrpE through an alteration of its conformation (6; Chapter 3). In order to detect a change in the relative conformation of DnaK325 in the presence and absence of ATP, trypsin digestion assays were performed as previously described (1).

As previously reported, and as can be seen in Fig. 5.5, DnaK undergoes a dramatic change in its conformation, going from what we commonly refer to as a "closed" conformation to a more "open" conformation in response to the presence of ATP (1). Surprizingly, DnaK325 undergoes

Figure 5.5. Trypsin digestion pattern of DnaK and DnaK325 in the presence and absence of ATP. 2.28μM DnaK325 (lanes 1 - 9) or DnaK (lanes 10 - 20) digested with 0.12μg Trypsin for various periods of time (in min) at room temperature without ATP (lanes 5 - 9 and lanes 15 - 20), or in the presence of 2.5mM ATP (lanes 1 - 4 and lanes 10 - 14).


DnaK325
DnaK+
ATP + + + + - - - - - + + + + + - - - - -
Time 30 20 15 10 30 20 15 10 0 30 20 15 10 0 30 20 15 10 0

no such change in conformation in the presence of ATP (Fig. 5.5). In the presence of ATP, the conformation of DnaK325 resembles that of DnaK in the absence of ATP, indicating that DnaK325 is either permamently found in the closed conformation, or may shift so rapidly between the open and closed conformations that the open conformation cannot be detected by trypsin digestion.

Interestingly, these results contrast with those found for another DnaK mutant protein, DnaK756, which appears to be always found in the open conformation (1). The fact that this protein is inactive and DnaK325 is active in λ DNA replication and in supporting bacterial growth may indicate that the closed conformation is more able to interact with substrate than the open conformation.

Discussion

The DnaK325 mutant was isolated and characterized genetically as being able to completely bypass the need for the *grpE* gene (5). Although it was isolated in the same selection and is structurally similar to the DnaK332 mutant, the results presented in this chapter indicate that the DnaK325 protein overcomes the need for GrpE in a manner that is different from the DnaK332 mutant and that more strongly resembles that of the DnaKc94 truncation mutant.

Like DnaKc94, DnaK325 appears to overcome the need for GrpE through changes in its conformation and an increase in

its ATPase activity. One significant difference between the DnaKc94 and DnaK325 mutant proteins, however, is that DnaKc94 does not completely eliminate the need for GrpE, and DnaK325 does. Furthermore, whereas DnaKc94 is unable to form a stable interaction *in vitro* with the λP protein, DnaK325 is able to do so. These results further support the ideas that GrpE plays a role in helping DnaK to interact with proper substrates (7), and that the abilities of GrpE to subtly alter the conformation of DnaK and to release nucleotide and substrate from DnaK have biological relevance.

It is implied from this work that the apparent inability of DnaK325 to undergo a conformational change in response to ATP affects its ability to interact with substrate. One possibility is that this apparent "stiffness" in conformation is simply due to the increased ATPase activity of the protein. In other words, it could be that DnaK325's inability to undergo a conformational change in the presence of ATP is due to its being rapidly recycled from the open to the closed conformation, thus leading to the observed higher ATPase activity. If this were the case, then DnaKc94, whose ATPase activity is even higher than that of DnaK325, would also be expected not to change conformation in the presence of ATP. However, this is not the case. When DnaKc94 is similarly digested in the presence and absence of ATP, its conformation changes in a

manner similar to that of wild-type DnaK (unpublished results). Therefore, it seems most likely that DnaK325 truly resides in a permanently "fixed," closed conformation.

One possible explanation for the toxic effect of GrpE on DnaK325's ability to function may lie in its fixed conformation. It may be that since DnaK325 has a reduced affinity for substrates, as exemplified by its slightly reduced affinity for the λP protein, the presence of GrpE may cause it to release substrates too quickly, thus inhibiting its chaperone activities.

The results presented in Chapter 4 indicated that GrpE may act as a trigger factor, responsible for maintaining a proper balance between the ATP- and ADP-bound, or nonactive and active forms of DnaK. The results presented here, along with previous results regarding the conformations of DnaK325 and DnaK756 build on that notion, and indicate that the particular conformation that DnaK adopts may be a more or less functional one.

It is somewhat surprizing to find that either a deletion of the carboxyl-terminus of DnaK (DnaKc94) or a single point mutation in the ATPase domain (DnaK325) have the same effect of increasing the ATPase activity of the protein. One might have expected to find that the ATPase activity of DnaK would not be altered by deletion of part of a domain normally thought to be separate from the ATPase

domain, or that mutation within the ATPase domain might inhibit or destroy the ATPase activity. While such mutations no doubt exist, it is not so surprizing that the mutations found in this work have a stimulatory effect on the ATPase activity if one considers the means by which they were selected. Both DnaK325 and DnaKc94 were selected for based on their abilities to compensate for an overall GrpE deficiency, and GrpE has the effect of stimulating the ATPase activity of DnaK. This means that DnaK's ATPase activity is designed to be higher than it normally is in the absence of its cofactors, and that the effect of GrpE on the ATPase activity is not a coincidental one, but rather a regulatory one which is important to wild-type DnaK's ability to function.

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CHAPTER 6

OVERVIEW AND PERSPECTIVES

There are now several examples in the biological world of protein chaperone systems, or sets of proteins that work in concert to help guide other proteins to their proper destinations, help with assembly and disassembly of protein structures, and help proteins to fold properly. The DnaK-DnaJ-GrpE system of *E. coli* is one example of such a system, the components of which have been extensively conserved in both the prokaryotic and eukaryotic worlds.

Over the past several years, it has become apparent that the DnaK protein is the major component of this system, responsible for interacting directly with substrates, and that DnaJ and GrpE function to help DnaK carry out its work, mainly through stimulation of DnaK's ATPase activity, alteration of DnaK's conformation and assistance to DnaK in locating proper substrates.

It was the goal of this dissertation to provide greater understanding of the role of the DnaK-GrpE interaction within this system. In the process, a few fortuitous results concerning the nature of the domains of the DnaK protein were also obtained. The following is a summary of the most important findings made during the course of this work.

(1) GrpE's stimulatory effect on DnaK's ATPase activity has biological relevance, and a model has been proposed for how such stimulation enables DnaK to function better.

(2) GrpE not only helps DnaK to release substrates, but helps it to interact better with them as well.

(3) In addition to stimulating the ATPase activity of DnaK, GrpE is able to subtly affect the conformation of DnaK, which may affect its ability to interact with substrates.

(4) The distinctions concerning the functional domains of DnaK are not as well deliniated as they were originally thought to be. Specifically (a) the carboxyl-terminal, supposed substrate binding domain of the protein is able to regulate the amino-terminal, ATPase domain of the protein; and (b) the carboxyl-terminal domain can be eliminated and the resulting truncated protein is still active, indicating that the amino terminus must form some interaction with substrate.

In light of the above findings, the model for the DnaK-DnaJ-GrpE system can be modified as follows. DnaK forms a stable interaction with substrates. Specific carboxyl-terminal domain sequences are necessary for the interaction between DnaK and substrate to be stabilized, as seen from the inability of DnaKc94 to form a stable interaction with λP.

DnaJ and GrpE are able to help DnaK to locate its substrates and possibly to help it form a more stable interaction, as seen by DnaJ's ability to help DnaK interact with σ^{32} and other previous work, and as implied

from this work regarding DnaKc94's inability to stably interact with λP and to replicate λDNA in the absence of GrpE.

The ability of DnaK to undergo a conformational change is also important for forming a stable interaction with substrate, as seen by DnaK325's fixed conformation and somewhat reduced affinity for substrate. It may be that this change in conformation is normally coupled to ATP binding or hydrolysis, but can become uncoupled through mutation, as exemplified by the properties of the DnaK756 and DnaK325 mutant proteins. It seems that the nucleotide-free, or "closed" conformation is the more able to interact with substrate since DnaK756 is fixed in the ATP-bound, or open conformation, and binds substrate very poorly, while DnaK325 is fixed in the closed conformation and is much better able to bind substrate than is DnaK756.

The changes that occur between DnaK's binding and release of substrate are still not well understood, although in most known functions, such as reactivation of heat inactivated RNAP, it has been found that ATP hydrolysis is required. The precise roles of DnaJ and GrpE in DnaK's actual chaperoning functions, and whether they are involved subsequent to substrate binding are unknown.

When DnaK is ready to release its substrate, GrpE specifically aids in this release by directly altering the conformation of DnaK which results in release of nucleotide

from DnaK. This in turn, aids in release of substrate. More specifically, it seems that release of ADP results in release of substrate, given DnaK332's ability to release ADP spontaneously, its ability to function in the presence of ATP and absence of GrpE, and its inability to release substrate in GrpE's presence.

Most likely, then, the stimulatory effect of DnaJ and GrpE on DnaK's ATPase activity works at several levels. It maintains a functional balance between the ATP and ADP bound forms of DnaK, which is tied to maintaining a proper balance between DnaK conformations. These balances are critical to DnaK's proper functioning as seen by the amino-terminal mutants' inherent alterations of these balances (DnaK325's inherently higher ATPase activity and DnaK332's spontaneous release of ADP), and the devestating effect of the presence of GrpE on their abilities to function, as exemplified by their inabilities to replicate bacteriophage λ DNA in the presence of GrpE.

As seen from this work, such alterations of balance can be achieved in a number of ways. Mutations in DnaK can overcome the need for GrpE either through changes in ATPase activity that can be achieved through deregulation at the amino-terminal or carboxyl-terminal ends of the protein, or through spontaneous release of ADP without any direct increase in the ATPase activity. They can also overcome the need for GrpE through direct alterations in their

abilities to change conformation that can be achieved by mutations in either the amino- or carboxyl-terminal regions as well.

Many homologs of the DnaK and DnaJ proteins are found in higher organisms. Although both types of proteins are able to perform chaperone functions, it is likely that the two still function together for some activities in eukaryotic systems as they do in *E. coli*. The GrpE protein has no other function in *E. coli* but that of aiding DnaK in its activities, and as there is no discovered homolog for GrpE in higher organisms, it would appear that the eukaryotic DnaK homologs have evolved so as to no longer require the services of GrpE. In this regard, the particular means employed in this work to elucidate the workings of the DnaK-DnaJ-GrpE machine may also shed light on the means by which higher organisms have eliminated the need for GrpE. The DnaK332 mutant is particularly interesting in this regard in that, while eliminating the need for GrpE, like the eukaryotic DnaK homologs, it retains a lowered ATPase activity. This particular mutation is at a highly conserved residue, so it cannot be part of the pathway by which the eukaryotic homologs have evolved. However, there may be other mutations in DnaK that are able to achieve similar results, which may mimick the eukaryotic homolog situation better, and which could potentially provide more insight both into the workings of

the DnaK-GrpE interaction and the functioning of the eukaryotic DnaK homologs as well.