Biochemical Characterizations of *Escherichia coli* DnaK and DnaK Mutant Proteins Purified from *ndk* Deficient Cells

by

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Bachelor of Arts in Biology
The Johns Hopkins University, 1989

SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOLOGY
AT THE
MASSACHUSSETTS INSTITUTE OF TECHNOLOGY

FEBRUARY 1999

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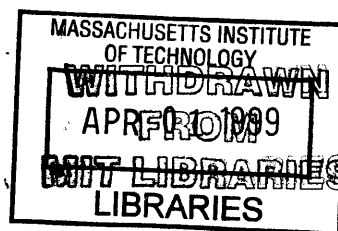
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February 3, 1999

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ABSTRACT

Preparations of DnaK purified in our lab as well as preparations of DnaK and other Hsp70 proteins obtained from other labs and providers were found to contain an ADP kinase activity and to produce an initial burst of ADP in ATPase assays. It was determined that both of these activities were due to the presence of a small amount of co-purifying nucleoside diphosphate kinase. The presence of this protein can explain various conflicting biochemical characterizations and novel properties reported for DnaK and Hsp70 proteins in the literature including the range of values for the k_{cat} of the ATPase reaction, initial burst kinetics of the ATPase reaction, and an ADP-ATP exchange activity by DnaK.

We detected a weak physical interaction between DnaK and nucleoside diphosphate kinase that could explain in part the co-purification of the proteins. Purification of DnaK from cells containing a disruption of the *ndk* gene led to the removal of most, but not all, of the ADP kinase activity from the DnaK preparations. Treatment of the DnaK to remove bound nucleotide resulted in the coversion of multiple forms of DnaK into one form, as detected by MonoQ chromatography, and permitted better separation of DnaK from the residual ADP kinase activity.

We purified DnaK derivatives with alterations at the threonine-199 and lysine-70 positions from *ndk* deficient cells using an extended protocol and were thus able to obtain preparations with greatly reduced amounts of co-purifying nucleoside diphosphate kinase. The biochemical characterizations of these two mutants led to the definition of two new classes of DnaK mutants. The DnaK T199S mutant has a near normal ATPase activity and undergoes an ATP induced conformational change, but is unable to fully function as DnaK in the cell. The DnaK K70A mutant has a defective ATPase activity and does not undergo an ATP induced conformational change. The characterization of these two mutants led us to formulate a novel model for the ATPase cycle of DnaK which involves the rapid and reversible hydrolysis of ATP.

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Acknowledgments

I am grateful for the sincere support and encouragement which my advisor Graham Walker has provided to me over the years. Graham has always shown true interest and enthusiasm for my work and has contributed greatly to my scientific development without ever being intimidating, patronizing, or unapproachable. I thank Graham for riding the roller coaster that has been my thesis project with me and for helping to smooth out the bumps.

True thanks go out to Marianne White for the invaluable help that she has provided to me and the lab as a whole during my time here. The benefit which she brings to our lab with all the good cheer and sweet treats cannot easily be measured.

I thank Peter Kim and Uttam RajBhandary for the helpful advice which they have given me from my preliminary examination all the way through to my last thesis committee meeting. I thank Frank Solomon and Cathy Squires for joining this group and taking the time out of their schedules to learn about and discuss my research.

I thank all of the members of the Walker lab, past and present, for making the lab such a supportive, stimulating, and fun place to work. I am grateful to my senior graduate students, who have now moved on, Melissa Lee, Sumati Murli, and Laura Willis, for welcoming me into the lab and providing support and camaraderie, and thank Greg York for going beyond the call of duty to provide help and keep the lab in working order. I enjoyed and benefited from the productive working relationship I had with J.D. Zhang while working on the DnaK project. I thank Gordon Campbell for ceaseless (good-natured) pestering and endless distractions during the writing of this thesis, and for never turning down the opportunity to go out for beers.

I thank the researchers in whose labs I have previously worked. I am grateful to Phillip Needleman and Amiram Raz at Washington University, Y.C. Lee and Kevin Rice at Johns Hopkins University, Chi Dang at Johns Hopkins Medical School, and Mich Hein at Scripps Research Institute for participating in my scientific development.

I am grateful to my family for supporting (almost all) of my pursuits in life and for never letting me feel as though my best effort wasn't good enough.

Last, but certainly not least, I thank Marci, my fiancee, for her love, support, and inspiration. I so look forward to being with you and the many happy years that lie ahead of us.

Chapter 1

Introduction

I. The DnaK Molecular Chaperone

A. The Hsp70 Family

The DnaK protein of *Escherichia coli* is a member of the highly conserved and ubiquitous Hsp70 class of proteins. Hsp70 proteins are molecular chaperones, proteins that bind to and stabilize an otherwise unstable conformer of another protein. Through controlled cycles of binding and release, molecular chaperones facilitate the correct fate of a protein *in vivo*, whether it be folding, oligomeric assembly, transport through cellular membranes, or degradation (45). Exposure of both prokaryotic and eukaryotic cells to a variety of physical stresses, including elevated temperature, results in the increased synthesis of a group of polypeptides called heat shock proteins. Hsp70s, or 70-kDa heat shock proteins, were originally identified as such proteins. Subsequently, Hsp70 proteins have been identified that are expressed constitutively and are not induced by heat shock. All Hsp70s have essential roles in protein metabolism under both stress and non-stress conditions. They are found in all prokaryotic cells and in most compartments of all eukaryotic cells (18). Eukaryotic cells have multiple members of the Hsp70 family, but DnaK is one of only two Hsp70 proteins in *Escherichia coli*, the other being Hsc66. Hsc66 is a Hsp70-like protein that shares less homology to other Hsp70s than does DnaK. Hsc66 does not complement loss of DnaK function, and its exact cellular function has yet to be determined (54, 93, 108)

B. Heat Shock Response

As a heat shock protein, DnaK provides an essential line of defense against stress by preventing the aggregation of and assisting the refolding of misfolded proteins. In *E. coli* there are at least 20 heat shock proteins, including DnaK, DnaJ, GrpE, GroEL, and GroES (69). Expression of the *E. coli* heat shock proteins is dependent on the formation of a complex between RNA Polymerase and σ^{32}, an alternative sigma factor encoded by the *rpoH* gene (39). The RNA Polymerase-σ^{32} complex specifically acts at the promoters of heat shock genes, which are not recognized by RNA Polymerase holoenzyme complexed with σ^{70} ($E\sigma^{70}$) (17, 39, 121).

Following a shift to higher temperature, the concentration and activity of σ^{32} increase markedly, resulting in a higher level of transcription at heat shock gene promoters (40, 99). This increase in σ^{32}-specific transcription results from the combined effects of increased stability, increased translation, and increased activity of σ^{32} (99, 105).

Several of the heat shock proteins, including DnaK, have been shown to be necessary for the regulation of σ^{32} activity and, consequently, for the regulation of the heat shock response (14, 76, 98, 104). DnaK is required for the negative regulation of σ^{32} during steady-state growth, and this repression is transiently lifted upon heat shock (11). DnaK directly interacts with σ^{32} (28, 58). Genetic experiments lead to the identification of a conserved regulatory region within σ^{32} called region C, spanning residues 122-144, which is required for DnaK dependent control of σ^{32} stability and synthesis (68). DnaK binds to peptides from a σ^{32} peptide library that are within region C (64). It is believed that DnaK either removes σ^{32} from its complex with RNA Polymerase leaving it susceptible to proteolysis or directs free σ^{32} to a proteolytic machinery (9). σ^{32} is rapidly degraded by the membrane-anchored protease HflB (46, 47, 107), resulting in the very short one minute half-life of σ^{32} under non-heat shock conditions.

Several models have been proposed to explain the mechanism by which DnaK modulates σ^{32} activity in response to physical stress. Carol Gross and others have proposed a "homeostatic model" in which denatured and misfolded peptides that result from thermal and physical stress serve as substrates for DnaK, titering DnaK away from σ^{32} and resulting in the stabilization of σ^{32} (19). This model is supported by the observation that unfolded peptides and abnormal accumulation of peptides result in the stimulation of heat shock gene expression (34, 80, 119). John McCarty has shown that the ATPase and autophosphorylation activities of DnaK are greatly stimulated as temperature increases and proposed that DnaK can serve as a "cellular thermometer" which directly senses environmental temperature and modulates its interaction with σ^{32} as a consequence of the level of its biochemical activities (65). Bernd Bukau has proposed a homeostatic model similar to that of Carol Gross, but assigned the key role of the "thermometer" to DnaJ (9).

C. Cellular Role of DnaK

While the expression of DnaK is increased by heat shock, DnaK, like other Hsp70 proteins, plays important roles in the cell under normal conditions. Cells lacking the *dnaK* gene are viable at 30°C, but the $\Delta dnaK$ cells display a number of abnormal phenotypes including slow growth, inability to grow at high or low temperature, and defective septation resulting in extreme filamentation of the cells (12, 13, 76). These *dnaK* deletion mutants and other *dnaK* mutants are defective in proper regulation of the heat shock response, resulting in abnormally high levels of heat shock protein expression at 30°C and a failure to turn off the response after a shift to 42°C (104). The $\Delta dnaK$ strain rapidly accumulates suppressor mutations that allow more rapid growth and restore proper septation, but the strain remains temperature sensitive (12). These suppressor mutations map to the *rpoH* gene and result in reduced activity of σ^{32} (14), suggesting that a primary role of DnaK at 30°C, as previously discussed, is to negatively regulate σ^{32} and the heat shock response. DnaK plays other important housekeeping functions. It is involved in the folding of nascent polypeptides (45, 88), translocation of proteins across the inner membrane (118), directing proteins for proteolysis (35), synthesis of flagella (94), maintenance of the negative supercoiling of DNA (73), and chromosomal segregation and plasmid maintenance (13, 106). DnaK is also required to disassemble protein complexes to allow the replication of bacteriophage λ (49, 74) and P1 (96, 113).

D. DnaK Biochemical Activities

DnaK, like other Hsp70s, has weak ATPase and autophosphorylation activities (123). Values for the precise rate of ATP hydrolysis by DnaK vary, but a value of about 0.05 molecules of ATP hydrolyzed per molecule DnaK per minute at 30°C is common in recent publications. The site of autophosphorylation of DnaK *in vitro* is the threonine residue at position 199 (65). This threonine residue is totally conserved and present in a highly conserved segment of all Hsp70s. Both the ATPase and autophosphorylation activities of DnaK are greatly stimulated by increasing temperature, increasing 400-fold and 70-fold respectively between 20°C and 50°C at a pH of 6.2

(65). The ATPase activity of DnaK is also stimulated by peptides (60) and by the heat shock proteins DnaJ and GrpE (59). The cycle of ATP hydrolysis results in the release of bound peptide by Hsp70, and the coordinated release of peptide resulting from cycles of ATP binding, hydrolysis, and ADP release is crucial for the proper functioning of all Hsp70s (10).

E. The Hsp70 Chaperone Machine

DnaK interacts directly with two other heat shock proteins, DnaJ and GrpE, in order to efficiently perform its proper function in the cell. The term "chaperone machine" refers to the functional unit created by the cooperation of these three proteins (33). Both λ and P1 DNA replication are fully efficient only when all three proteins are present (49, 74, 96, 106, 113). The three proteins have also been shown to act together during the processes of protein folding (57), the refolding of denatured proteins (37, 92), and the modulation of the synthesis and stability of σ^{32} (9, 28, 98). DnaJ-related chaperones are found in all Hsp70 containing compartments of prokaryotic and eukaryotic cells. They are a heterogeneous group of multidomain proteins defined by a highly conserved domain of approximately 80 amino acids called the "J-domain" (55), which is often located near the amino terminus. DnaJ stimulates ATP hydrolysis by DnaK (59, 63). Other DnaJ proteins increase the rate of ATP hydrolysis of their corresponding Hsp70, and the J-domain is responsible for this stimulation (53, 100, 109). The solution structures of the J-domain of two family members show that it consists of four α-helicies with a loop between helicies 2 and 3 containing a conserved sequence motif (HPD) which is implicated in the interaction of the J-domain with Hsp70 (82, 85, 101, 109). Unlike DnaJ family proteins, GrpE proteins are found only in prokaryotes and in the mitochondria of eukaryotic cells (21, 67). GrpE accelerates the nucleotide exchange of DnaK by reducing the affinity of DnaK for ADP (75). GrpE interacts with the amino-terminal ATPase domain of DnaK (6), and the crystal structure of the stable complex between amino-terminally truncated GrpE and the ATPase domain of DnaK has been solved (44). Together, DnaJ and GrpE stimulate the ATP turnover rate of DnaK by at least several hundred fold (63).

F. Conserved Hsp70 Structure/Function

Hsp70 proteins have been remarkably conserved throughout evolution with *E. coli* DnaK showing approximately 50% homology at the amino acid level to human and *Drosophila* Hsp70 (3). Hsp70 proteins are structurally subdivided into three domains: a highly conserved amino-terminal ATPase domain of ~44 kDa, followed by a conserved substrate binding domain of ~15 kDa, and finally a less conserved carboxy-terminal domain of ~10 kDa. The three-dimensional structures of the ATPase domains of bovine Hsc70 (23), human Hsp70 (97), and *E. coli* DnaK (44) are nearly identical and are very similar to the protein fold of actin and hexokinase (24). The ATPase domain consists of two globular subdomains separated by a deep central cleft and connected by two crossed α-helicies. ATP, coordinated by one Mg^{2+} and two K^+ ions, is bound at the bottom of the cleft by interactions with both subdomains and the connecting helicies. The cycles of ATP binding and hydrolysis in the ATPase domain of Hsp70 proteins control conformational changes in the protein that result in peptide binding and release, but the mechanism coupling the two processes is poorly understood. The chemical energy of ATP is used to perform mechanical work, the opening and closing of the substrate binding pocket, leading to low and high affinity forms of Hsp70 for peptide substrate. Several studies suggest that ATP binding triggers an association of the ATPase domain with the substrate binding domain, causing further conformational changes within the substrate binding domain itself (7, 43, 114). The crystal structure of the substrate binding domain of DnaK (residues 389-607) has been solved and is composed of the β-structured polypeptide binding site and a latch-like subdomain of 5 α-helicies (122). The co-crystallized heptapeptide, shown to bind to DnaK with high affinity (36), is fixed in a channel formed mainly by loops protruding from the bipartite eight-stranded anti-parallel β-sheet. The peptide is bound in an extended conformation as suggested previously by NMR experiments (56). The interaction between peptide and chaperone is dominated by hydrophobic interactions, confirming previous biochemical studies of the substrate specificity of Hsp70s (27, 36, 89). The α-helical subdomain is not in direct contact with the bound peptide, but it may work as a lid to "open" and "close" the substrate binding site. Peptide substrate with an attached photoactivatable

group photocrosslinks to residues of DnaK in the peptide binding site in a manner consistent with the deduced crystal structure (120).

II. The Functional Cycle of DnaK

The nature of the functional cycle of DnaK and other Hsp70 proteins has been the subject of intensive investigation. Once it became established that the cycle of ATP binding, ATP hydrolysis, and nucleotide release by DnaK/Hsp70 is linked to the cycle of peptide binding and peptide release by DnaK/Hsp70, the focus shifted to defining the steps of the functional cycle in detail. The majority of recent publications support a model of the functional cycle of DnaK which is described below and which is depicted in Figure 1. A discussion of the experimental evidence which was used to build the model follows. This model specifically describes the functional cycle of DnaK, but it is nearly identical to the prevailing model for Hsp70 function.

A. The Model

Conformational changes in DnaK drive its cycles of peptide binding and release. These conformational changes require the binding and hydrolysis of ATP and are regulated by the chaperone cofactors DnaJ and GrpE. DnaK alternates between two key conformational states: an ATP-bound state which has low affinity for peptide substrate and an ADP-bound state which has high affinity for peptide substrate. In the absence of peptide or cofactors the rate-limiting step of the ATPase cycle of DnaK is ATP hydrolysis. DnaJ, which itself binds peptides, recruits substrate to DnaK in its low affinity, ATP-bound form. DnaJ stimulates the hydrolysis of ATP by DnaK, which simultaneously brings DnaK to its high affinity, ADP-bound conformation. The change from low affinity to high affinity DnaK is due to a global conformational change driven by ATP hydrolysis which closes the lid of the peptide binding site. When DnaK is stimulated by DnaJ, the rate-limiting step of the ATPase cycle becomes ADP release. GrpE stimulates ADP release by DnaK. ATP now quickly binds to DnaK, triggering a change back to the conformation with low affinity for peptide. Thus, ATP binding results in peptide release. ATP binding occurs in two

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steps: a fast binding of the ATP followed by a slower conformational rearrangement which results in peptide release. The cycle can now repeat using the same or a different peptide as a substrate.

B. Experimental Evidence

1) ATP Hydrolysis Is Rate-Limiting

The steady-state ATPase rate of DnaK is very slow with reported k_{cat} values of 0.02-1.0 min.$^{-1}$ (see "**IV. Discrepancies**" below). Many researchers have concluded that in the absence of cofactors and peptide, hydrolysis of ATP to ADP is the slow step in the ATPase cycle of DnaK and all Hsp70s and is responsible for the low k_{cat} value. If the assumption is made that hydrolysis is non-reversible, then the ratio of ADP to ATP bound to DnaK/Hsp70 provides an indication of whether ATP hydrolysis or ADP/ATP exchange is rate-limiting. A ratio of ATP/ADP bound to DnaK/Hsp70 in steady-state hydrolysis of greater that 1.0 provided evidence that hydrolysis is rate-limiting (30, 63). Several groups made the assumption that the rate of ADP formation under single turnover conditions is identical to the rate of ATP hydrolysis. Finding that the single turnover rate of ATP is the same as the steady-state rate of ATP hydrolysis, the conclusion was made that both are governed by the same rate-limiting step, which must be ATP hydrolysis (42, 90, 103).

2) ATP Binding, Not ATP Hydrolysis, Causes Peptide Release

The cycle of peptide binding and release is stochiometrically coupled to the hydrolysis of ATP (81). Initially, it was believed that ATP hydrolysis is required to trigger the conformational change in DnaK from the form with high affinity for peptide substrate to the form with low affinity for peptide substrate. Thus ATP hydrolysis was modeled as the step of the ATPase cycle that leads to dissociation of the DnaK·peptide complex or "peptide release" by DnaK. This model was based on the observation that ATP, but not the nonhydrolyzable ATP analog AMP-PNP, causes DnaK to both release a model peptide substrate and to undergo a conformational change as

visualized by partial trypsin digestion (60). AMP-PNP acts as a competitive inhibitor of ATP, showing that it binds to the same site as ATP on DnaK. It was shown that other nonhydrolyzable ATP analogs, including ATPγS and AMP-PCP, also fail to induce peptide release or a conformational change by DnaK (79, 91). More recently, however, a general consensus has been reached that ATP binding and not ATP hydrolysis is responsible for both peptide release and the conformational change of DnaK. Anthony Fink's group showed that ATP induces mutant DnaK T199A, which has a much slower ATPase rate than wild-type DnaK, to release peptide. Assuming that ATP hydrolysis, and not some other step of the ATPase cycle is defective in this mutant, Fink concluded that ATP hydrolysis cannot be responsible for peptide release (78). This group also showed that ATP results in the relatively rapid release of peptide (less than the 12 minutes required to run a sizing column), and cannot be caused by ATP hydrolysis, assuming that ATP hydrolysis is the slow step of the ATPase cycle (78). Other groups also concluded that ATP binding and not ATP hydrolysis is required for peptide release by DnaK/Hsp70. It was shown that DnaK T199A undergoes a conformational change upon the addition of ATP (7). Again assuming that it is ATP hydrolysis that is defective in the DnaK T199A mutant, the conformational change, like peptide release, must be a result of ATP binding. BiP, a Hsp70 of the endoplasmic reticulum, with a T229G mutation (residue 229 of BiP is homologous to residue 199 of DnaK) also undergoes a conformational change and releases peptide upon the addition of ATP, again leading to the conclusion that ATP binding is responsible for both activities (111). Kinetic measurements have been made using stopped-flow apparatus of the release of fluorescently labeled peptide from DnaK/Hsp70 and the rate of DnaK/Hsp70 conformational change upon the addition of ATP. These measurements show that both rates are too fast to be accounted for by the slow single turnover rate of ATP hydrolysis and therefore must be due to ATP binding (22, 43, 83, 95, 103). The rates of peptide release and conformational change are very similar, supporting the model that the conformational change of DnaK results in peptide release, and that the functional activity of DnaK requires conformational changes (52).

3) ATP Binding Occurs in Multiple Steps

The kinetics of the conformational changes of DnaK/Hsp70 can be measured by changes in the fluorescence of tryptophan, of which DnaK has a single residue. Data from stopped-flow experiments shows that ATP binds to DnaK in a reaction that has at least two, perhaps three, steps. These include a rapid initial binding of the ATP followed by a slower rearrangement which leads to the conformational change associated with peptide release (22, 43, 95, 103). According to the interpretation of these observations, ATP hydrolysis follows the final binding rearrangement.

4) Changes in On and Off Rates Cause Peptide Release

The affinity of DnaK for peptide is about 40 times higher in the absence of ATP than in its presence (91). Kinetic measurements of the on- and off-rates of a fluorescently tagged peptide show that ATP increases the on-rate 47-fold, while it increases the off-rate 440-fold, resulting in an overall weaker affinity for peptide (91). The authors of this study indicate that ATP hydrolysis is not the slow step of the ATPase cycle, that DnaK-ADP binds peptide weakly, and that the peptide binding-release cycle of DnaK is not stochiometrically coupled to the hydrolysis of ATP (91). However, as previously discussed, other groups have argued that ATP hydrolysis is the slow step of the ATPase cycle, that DnaK-ADP binds peptide with high affinity, and that the peptide binding-release cycle of DnaK is stochiometrically coupled to the hydrolysis of ATP. Peptide binding occurs in a two step process in the presence of ADP and in a one step process in the presence of ATP (102). The observations that the nucleotide-bound state of the N-terminal domain of DnaK/Hsp70 affects the peptide-binding affinity of the C-terminal domain and that the presence of peptide stimulates the ATPase rate indicate that there is communication between the two domains (7, 102).

5) DnaJ and GrpE

As previously discussed, the heat shock proteins DnaJ and GrpE are required for DnaK to perform many of its functions. The steady-state turnover rate of unstimulated ATPase by DnaK is

too slow to drive its chaperone activities, even in the presence of substrates, which stimulate the ATPase activity 2-10 fold (26, 31, 42, 51, 63, 103). It is therefore essential that regulatory mechanisms exist to increase ATP turnover and chaperone function.

DnaJ stimulates the rate of DnaK ATPase by accelerating the rate of ATP hydrolysis (59, 63). In addition to binding to DnaK, DnaJ binds to substrates of DnaK with kinetics fast enough to prevent their aggregation (29, 57, 92). By using partial DnaJ fragments, it was shown that stimulation of DnaK by DnaJ involves two signals, one which is mediated by the J-domain and another that is provided by substrate (53). Thus, when the DnaJ-substrate complex binds to DnaK-ATP, the substrate is transferred into the DnaK binding pocket with the subsequent stimulation of ATP hydrolysis by the J-domain. This mechanism tightly couples substrate binding and ATP hydrolysis (84). DnaJ rapidly brings DnaK into the high substrate affinity DnaK-ADP form, and due to the slow rate of hydrolysis, DnaK does not go through futile rounds of ATPase activity in the absence of DnaJ stimulation. The binding site for the J-domain on DnaK is not known. The ternary complex between DnaK, DnaJ, and substrate is unstable (29, 110) and DnaJ leaves the complex immediately after ATP hydrolysis. DnaJ acts catalytically in targeting substrates to DnaK (61). The degree to which DnaJ stimulates the ATPase rate of DnaK varies from report to report, but is likely in the range of 10-20 fold.

ADP release by DnaK is about 10-20 times faster than the rate of hydrolysis in the unstimulated ATPase cycle, but it becomes rate-limiting in the DnaJ-stimulated cycle (63). GrpE acts as a nucleotide exchange factor and accelerates the rate of dissociation of ADP from DnaK about 5000-fold (75). The crystal structure of the GrpE dimer and ATPase domain of DnaK shows that GrpE stimulates ADP release by opening the nucleotide binding cleft of DnaK with a 14° rotation of the IIB subdomain (44). Once ADP is released, ATP rebinds to DnaK with fast kinetics ($k_{on} = 90$ min.$^{-1}$) (103). The rebinding of ATP triggers the release of bound substrate. The substrate can now either go through another round of DnaK mediated folding, be transferred to the GroEL/GroES chaperone machine, or achieve its native conformation. Thus, DnaJ and

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GrpE stimulate the functional cycle of DnaK in a controlled manner and together stimulate the rate of steady-state ATPase by DnaK at least several hundred-fold (63).

III.   Structural Basis of DnaK/Hsp70 ATPase Activity

The x-ray crystallographic structures of the ATPase fragments of bovine Hsc70 (23), human Hsp70 (97), and *E. coli* DnaK (44) have been solved. All three structures are nearly identical and are very similar to the three-dimensional structures of actin and hexokinase, despite negligible similarity between the Hsp70s and actin and hexokinase at the level of amino acid sequence (24). Optimal superposition of structures and alignment of sequences lead to the definition of an ATPase domain superfamily to which prokaryotic cell cycle proteins, sugar kinases, actin, and Hsp70 proteins belong (5). Within the nucleotide binding site of Hsc70 is a Mg^{++} ion that is octahedrally coordinated by oxygens from water molecules and phosphate groups of the bound nucleotide. Amino acid side-chains of several residues interact with the metal-nucleotide complex in the active site including carboxylates of D10, E175, D199, and D206, the ε-amino group of K71, and the hydroxyl group of T204 (25). These amino acids are conserved in all Hsp70s and correspond to D8, E171, D194, D201, K70, and T199 of *E. coli* DnaK. Refinement of the Hsc70 structure to 1.7 angstroms reveals two K^+ ions in the nucleotide binding cleft which are also involved in specific interactions and act as specific metal cofactors in the ATPase reaction of Hsc70 (116). Potassium is required by Hsc70 for optimal ATPase activity, and substitution of K^+ with other monovalent cations results in lower rates of ATP hydrolysis (72). This observation is consistent with the report that both K^+ and ATP are required for peptide release by DnaK (78).

Hsp70 proteins have an autophosphorylation activity and the autophosphorylated residue of *E. coli* DnaK was determined to be threonine-199 (65). Mutation of this threonine to alanine or valine greatly reduces the ATPase activity (65) and leads to a DnaK protein that cannot properly function *in vivo* (66). The speculation was made that the mechanism of hydrolysis of Hsp70 proteins involves an in line attack by the hydroxyl group of this conserved threonine on the γ-

phosphate of the bound ATP. However, mutation of T204 of Hsc70 to valine only lowers the ATPase rate a few fold, and solution of the structure of the Hsc70 T204V mutant lead to the conclusion that T204 is not an obligatory intermediate phosphate acceptor in the ATP hydrolysis reaction (71). Mutation of residues D10, E175, D199, or E206 in a 44-kDa amino-terminal fragment of Hsc70 all result in proteins with reduced, but not abolished ATPase activities (115). It was postulated that no single residue of Hsp70 is catalytically indispensable for ATP hydrolysis, until it was shown that mutation of Hsc70 lysine-71 to glutamic acid, methionine, or alanine results in a protein with no detectable ATPase activity (70). This data, together with the crystal structures of the mutant proteins determined to a resolution of 1.7 angstroms, identified lysine-71 as a residue that is essential for the chemical hydrolysis of ATP (70). The proposal that the ε-amino side chain of K71 stabilizes an H_2O molecule or an OH^- ion for a nucleophilic attack on the γ-phosphate of ATP had previously been made (25, 115).

A number of other mutants of DnaK/Hsp70 proteins have been characterized, providing insight into the mechanisms by which the proteins function. A set of mutations of DnaK was isolated by selecting for cells with high constitutive expression of heat shock proteins (defective in σ^{32} regulation). The mutations all changed amino acids which are highly conserved among the Hsp70 family: T154, E171, A174, D201, G229, and G341 (117). Of the mutations, D201N and E171K do not undergo a conformational change upon the addition of ATP (52). Bernd Bukau's group also found that mutations at E171 do not undergo a conformational change, but do retain ATPase activity (although the ATPase activity varies widely between different mutants; see "**IV. Discrepancies**" below). They conclude that residue E171 is required for the coupling of the ATPase activity with substrate binding and release (8). One group reported that mutation of residue D10 of Hsc70 results in the loss of all ATPase activity and concluded that aspartate-10 is essential for ATPase activity (50). This result, however, is in disagreement with the results of David McKay's group (115) (see above and see "**IV. Discrepancies**" below). Mutation of residues T37, T229, and E201 of BiP, corresponding to T11, T199, and E171 of DnaK, produce proteins that have an ATPase activity reduced to less than 4% that of wild-type BiP and are

deficient in peptide release (32). When residues G226 and G227 of BiP (homologous to G196 and G197 of DnaK) are mutated, the affinity for ATP is lowered (112). Mutation of proline-143 of DnaK to serine lowers the ATPase rate of the protein and increases the amount of peptide required to stimulate the ATPase activity (15). Residue D10 of Hsp70 (D8 of DnaK) is required for the effect of ATP and ADP on peptide binding, and residue K71 of Hsp70 (K70 of DnaK) is required for the effect of ATP on peptide binding (86). Mutation of residue E543 of Hsc70 relieves inhibition by the peptide binding domain on the ATPase rate, resulting in a higher ATPase rate than wild-type in the absence of peptide (41).

IV. Discrepancies

In the 15 years since it was reported that DnaK possesses an ATPase activity (123), a wide range of quantitative and qualitative descriptions of the ATPase activity of DnaK and other Hsp70 proteins have been given. Standard conditions for ATPase assays of DnaK are 30°C, pH ~ 7.6, $[K^+]$ ~ 50 mM, $[Mg^{++}]$ ~ 10 mM and are not responsible for the discrepancies described below. The ATPase activity of unstimulated DnaK has been assigned k_{cat} values ranging from 0.02 - 1.0 min.$^{-1}$ (4). The general trend in the value reported for k_{cat} is towards lower numbers. The turnover rate of ATP by DnaK was initially reported at 1.0 min.$^{-1}$ (123), and later reports agreed with this value (16, 87). However, the values reported for k_{cat} began to fall. For example, a k_{cat} of 0.06 min.$^{-1}$ was reported in 1991 (65) and a k_{cat} of 0.087 min.$^{-1}$ was reported in 1993 (77). The authors of these two studies postulated that the removal of contaminating ATPases, such as GroEL and peptides, which stimulate DnaK ATPase, by more rigorous purification protocols, which include size exclusion chromatography and high resolution anion-exchange chromatography, lead to the lower k_{cat} values. Recently, k_{cat} values as low as 0.018 min.$^{-1}$ have been reported (83), although slightly higher values of 0.04 - 0.05 min.$^{-1}$ are more common (15, 51, 53, 63), and even higher values of 0.13 min.$^{-1}$ were reported recently (20, 91). Values reported for the degree of stimulation of DnaK ATPase by DnaJ, GrpE, and peptide also vary widely. For example, DnaJ and GrpE together have been reported to stimulate the rate of DnaK

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ATPase anywhere from 50-fold (59) to 1600-fold (62). Further complicating matters, the ATPase rate of DnaK can vary by several fold or more from preparation to preparation within the same lab (51, 90) and (Bernd Bukau, personal communication). As a general rule, researchers have considered the preparations of DnaK which have the higher ATPase activities to have "anomalous ATPase activities" (90) due to a small amount of contaminating ATPase or peptide and discard them in favor of preparations that have the lowest ATPase activity.

In addition to the wide range of values reported for the rate of the ATPase reaction of DnaK, the K_m values given for the ATPase reaction vary from 20 nM (15) to 4 μM (8). One possible cause for this large discrepancy is the phenomenon of mutual depletion kinetics. One assumption used to derive the Michaelis-Menten equation for irreversible single substrate reactions is that the concentration of free substrate is much greater than the concentration of the enzyme-substrate complex, or [S] >> [ES]. Since DnaK binds to ATP with very high affinity, yet has a very low turnover rate, certain combinations of DnaK concentration and ATP concentration exist in which the formation of the DnaK-ATP complex significantly depletes both free DnaK and free ATP. Under these "mutual depletion" conditions, [S] is not much greater than [ES], and the Michaelis-Menten equation is not valid (38). One way to avoid this complication is to keep the concentration of enzyme significantly lower than the concentration of substrate, so that the concentration of substrate remains much greater than the concentration of the enzyme-substrate complex. Adhering to this precaution results in the determination of lower K_m values (15). However, mutual depletion effects have been commonly ignored in kinetic characterizations of DnaK and other Hsp70s, even in recent publications, resulting in the determination of high K_m values. It should be noted that mutual depletion effects lead to erroneously high K_m values, but do not effect V_{max} or k_{cat} values. The depletion of free substrate lowers velocity measurements at low substrate concentrations, raising the value determined for K_m. However, as the substrate concentration is increased, the assumption [S] >> [ES] once again holds, and an accurate value is determined for V_{max}.

Several recent reports differ from the consensus description of the biochemical activity of DnaK in more general ways. As previously discussed, most researchers have concluded that the hydrolysis of ATP is the rate-limiting step in the ATPase cycle of DnaK. However, Maciej Zylicz's group reported that the hydrolysis of ATP occurs with an initial burst of ADP production followed by a slower steady-state rate of hydrolysis, indicating that some step following ATP hydrolysis (the group concludes that it is the dissociation of P_i and ADP) is rate-limiting (1, 2). The current model of the DnaK ATPase cycle states that the hydrolysis of ATP is not a reversible step. However, Hiroshi Kido's group reports that DnaK is able to phosphorylate ADP to ATP, indicating that hydrolysis is reversible (48). The authors argue that nucleoside diphosphate kinase is not a contaminant in their DnaK preparations and that the ADP kinase activity that they observe is intrinsic to DnaK. The ATPase activity of DnaK was also reported to be autostimulatory, that is that higher concentrations of DnaK were measured to have a higher rate of ATPase per DnaK molecule than lower concentrations of DnaK (87). The observation of the autostimulation of DnaK, however, was later refuted (77).

The kinetic characterization of certain DnaK/Hsp70 mutants has not been consistent. For example, David McKay's group reported that mutation of lysine-71 of the 44-kDa amino-terminal fragment of Hsc70 to glutamic acid, methionine, or alanine leads to a protein with no ATPase activity and concluded that lysine-71 is essential for hydrolysis (70). However, another group found the K71E mutant of Hsp70 to have a measurable ATPase activity that is reduced to 0.08 times the rate of wild-type Hsp70 (86). This group (86) as well as McKay's group (115) found that mutation of D10 preserves ATPase activity, in contrast to a group that found D10 to be essential for ATPase activity (50). The ATPase rate of the mutant DnaK756 was originally found to be lower than that of wild-type DnaK (123), but was later measured by the same group to be 50-fold higher than wild-type (59). Other mutations of DnaK and Hsp70 have measured kinetic values that vary significantly from lab to lab. Also, different mutations of the same amino acid residue, such as E171 of DnaK (8), have ATPase rates which differ significantly, even when measured in the same lab.

A paradox exists with the model for the functional cycle of DnaK described previously. The model states that ATP binding, and not ATP hydrolysis, results in the conformational change of DnaK that causes peptide release. However, the nonhydrolyzable analogs of ATP, AMP-PNP, AMP-PCP, and ATPγS all fail to induce peptide release or a conformational change by DnaK (60, 79, 91), even though they act as competitive inhibitors of ATP. The apparent contradiction that ATP binding results in peptide release, yet binding of nonhydrolyzable analogs does not result in peptide release is resolved by postulating that the nonhydrolyzable analogs do not bind to DnaK with the exact geometry required to effect a conformational change. However, the crystal structure of AMP-PNP complexed with the ATPase fragment of Hsc70 was determined to a resolution of 2.4 angstroms and found to resemble the structure of Hsc70 complexed with ADP + P_i so closely that it was used as a model for the prehydrolysis binding of ATP to Hsc70 (25). Thus, the difference between the binding of the nonhydrolyzable analogs of ATP and the binding of ATP itself to DnaK/Hsp70 is subtle, but must be significant if ATP binding plays the role of conformational effector as described in the consensus model.

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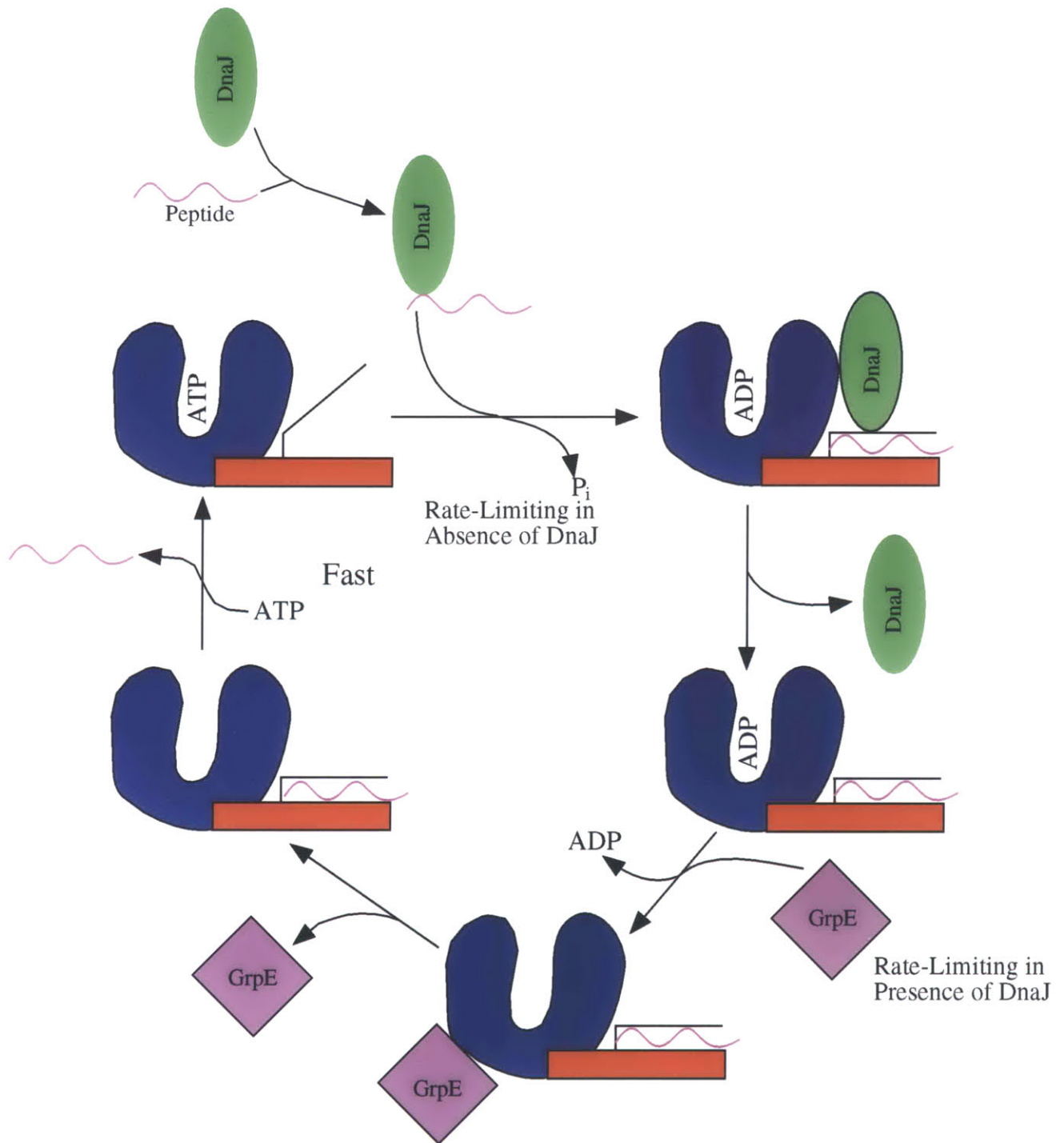
Figure 1. Model for the Functional Cycle of DnaK

DnaK is represented by the object: (low energy, low peptide affinity) and

 (high energy, high peptide affinity), where blue is the ATPase domain and red is the peptide binding domain. See text for details.

Chapter 2

DnaK and Hsp70 Preparations Contain Initial Burst and ADP Kinase Activities
That Are the Property of a Co-Purifying Protein

Abstract

Preparations of *Escherichia coli* DnaK from our lab as well as preparations of DnaK and other Hsp70 proteins from several major labs in the field produce a stoichiometric initial burst of ADP when incubated with ATP and contain an ADP kinase activity. The presence of these activities initially suggested to us a new model to describe the ATPase activity of DnaK and the mechanism by which DnaK functions as a molecular chaperone. However, we determined that the initial burst activity results from the transfer of gamma-phosphate from substrate ATP to ADP bound by the DnaK and is the same activity that results in ADP phosphorylation. The kinase activity that is responsible for both the initial burst of ADP formation and the phosphorylation of ADP is able to phosphorylate CDP as well as it phosphorylates ADP, and is therefore nonspecific with regard to the nucleoside diphosphate substrate. The ADP kinase activity found in preparations of the 44-kDa amino-terminal ATPase fragment of *Thermus thermophilus* DnaK transgenically expressed in *E. coli* is abolished at high temperatures, showing that it is associated with a copurifying protein from *E. coli*. We conclude that the ADP kinase activity present in DnaK and Hsp70 preparations is also the property of a very low amount of a copurifying protein that is most likely nucleoside diphosphate kinase. The presence of this protein can explain many of the discrepancies in the literature regarding the characterization of the ATPase activity of DnaK as well as recent reports of initial burst kinetics by DnaK (Banecki, B. and Zylicz, M. (1996) *J. Biol. Chem.* 271, 6137-6143) and an ADP-ATP exchange activity of DnaK (Hiromura, M., Yano, M., Mori, H., Inoue, M., and Kido, H. (1998) *J. Biol. Chem.* 273, 5435-5438).

Introduction

DnaK is the principal *Escherichia coli* member of the highly conserved and ubiquitous family of Hsp70 proteins. In addition to functioning in response to heat shock and other forms of stress, many Hsp70 proteins, including DnaK, function in a variety of normal metabolic processes, including the folding of nascent polypeptides (26, 43), intracellular protein trafficking and membrane translocation (11), protein degradation (50), and disassembly of native protein complexes (54). Hsp70 proteins function as molecular chaperones in all of these processes, undergoing multiple cycles of binding and release of the polypeptide substrate in order to facilitate its folding or to protect unfolded states from aggregation and denaturation (8, 12, 20, 24, 25). DnaK acts as a molecular chaperone in *E. coli*. *In vitro*, DnaK blocks the aggregation of and allows the reactivation of heat-inactiviated RNA polymerase (48), rhodanese (31), and firefly luciferase (47). DnaK dissociates dimeric RepA protein to allow P1 phage replication (55) and promotes the disassembly of the protein complex at *ori-λ*, allowing replication (28). DnaK is required for growth at high temperature (39) and is required for the negative regulation of the heat shock response at normal growth temperatures (52). DnaK represses the expression of heat shock genes by negatively regulating the synthesis and stability of σ^{32} (9, 50), and this repression is transiently lifted upon heat shock (7).

Limited proteolysis of Hsp70 proteins separates them into two stable domains. The C-terminal domain binds peptides in an extended conformation (30, 58) in a manner believed to mimic the binding of physiological substrates. The N-terminal domain possesses the weak intrinsic ATPase activity associated with every Hsp70 protein (15, 23). The two domains interact, and the peptide binding and release cycle is tightly coupled to the ATP binding and hydrolysis cycle (2, 8, 20, 25). The ATPase activity of Hsp70 proteins is therefore crucial for their biological function.

A range of quantitative values and functional descriptions of the ATPase activity of DnaK and other Hsp70 proteins have been reported. The ATPase activity of unstimulated DnaK has been

assigned k_{cat} values ranging from 0.02 - 1.0 min.$^{-1}$ (4, 42, 59) and K_m values ranging from 20 nM - 4 μM (6, 10). The binding of ATP, but not nonhydrolyzable analogs of ATP, results in a conformational change of DnaK and in the release of its bound peptide substrate (33), leading to the conclusion that ATP hydrolysis results in a global conformational change of DnaK which in turn results in peptide release. However, because mutants of DnaK that have a defective ATPase activity release peptide (40) and undergo a conformational change (5) upon ATP binding, it was concluded that ATP binding, and not hydrolysis, results in a conformational change of DnaK that results in peptide release. Measurements of the amounts of ATP and ADP bound to DnaK during ATP hydrolysis (34) and single ATP turnover rates (44, 51) led to the conclusion that the hydrolysis of ATP is the rate-limiting step in the ATPase reaction cycle of DnaK and cannot be required for the conformational change of DnaK and the consequent peptide release, both of which occur rapidly when ATP is added to DnaK (14, 42, 49, 51).

We found that DnaK and Hsp70 preparations display initial burst kinetics in ATPase assays, rapidly producing a stoichiometrically equal amount of ADP when incubated with ATP. We also found that these preparations contain an ADP kinase activity, phosphorylating ADP to ATP in the presence of ATP. These results were unexpected, because they could have indicated that ATP hydrolysis is a rapid and reversible step in the ATPase cycle of DnaK, a notion contrary to the current consensus in the literature. The results also allowed us to postulate a model for the functional cycle of DnaK, significantly different than the current model, and which resolved the paradox that ATP binding, but not the binding of nonhydrolyzable analogs of ATP, cause the conformational change which results in peptide release by DnaK. However, in the course of testing this model we found that the initial burst and ADP kinase activities are associated with a copurifying protein present amounts which we could not detect in the DnaK and Hsp70 preparations. The presence of this protein accounts for results of two papers published during the course of this study that also differ with the consensus characterization of the DnaK ATPase activity (3, 27).

Materials and Methods

Reagents and Media. ATP and ADP were purchased from Sigma Chemical Co. (St. Louis, MO). $[\alpha\text{-}^{32}P]$ATP, $[\gamma\text{-}^{32}P]$ATP, $[8\text{-}^{14}C]$ADP, and $[2\text{-}^{14}C]$CDP were purchased from DuPont NEN Life Science Products, Inc. (Boston, MA). Ampicillin, tetracycline, and chloramphenicol were purchased from Sigma. Potassium chloride, magnesium chloride, sodium chloride, lithium chloride, ammonium sulfate, EDTA, tris, hydrochloric acid, sucrose, and glycerol were purchased from Mallinckrodt Chemical, Inc. (Paris, KY), 2-mercaptoethanol, dithiothreitol (DTT), ovalbumin, and spermidine were purchased from Sigma, Hepes was purchased from USB (Cleveland, OH), magnesium acetate was purchased from Matheson, Coleman, & Bell (Los Angeles, CA), and formic acid was purchased from Fisher Scientific (Fair Lawn, NJ). LB liquid media was as described (45). Antibiotics were used at the following concentrations: ampicillin (100 μg/ml), tetracycline (12.5 μg/ml), and chloramphenicol (30 μg/ml).

Hsp70 Proteins. Hsp70 proteins were generously supplied to us from the following sources with references to the method of purification: *Escherichia coli* DnaK was provided to us by the laboratories of Bernd Bukau (34), Carol Gross (56), and Roger McMacken (29), 44-kDa N-terminal recombinant *Thermus thermophilus* DnaK was provided to us by the laboratory of Andrzej Joachimiak (38), recombinant human Hsp70 was provided to us by the laboratory of Richard Morimoto (17), and recombinant hamster BiP was provided to us by the laboratory of Linda Hendershot (53). Bovine Hsc70, purified from bovine brain cells, was purchased from StressGen Biotechnologies Corp. (Victoria, B.C., Canada).

DnaK Purification. The standard protocol for the purification of DnaK was similar to the method previously described (35) with some modifications and was as follows. GW8320 (MC4100, *ΔdnaK52 sidB1*) cells (35) carrying the DnaK overexpressing plasmid pJM6 (pBS-P$_{lac}$*dnaK*) (35) were grown at 30°C to late log phase (A$_{600}$ ~ 0.6) in 2 liters of LB media with

ampicillin, tetracycline, and chloramphenicol. Expression of DnaK was induced by adding IPTG (1 mM) and the culture was shifted to 37°C and incubated for another 1 hour. The cells were pelleted by centrifugation (5000 x g, 30 minutes, 4°C). The cell pellet was resuspended in 6 ml Buffer L1 (10% sucrose, 50 mM Tris-HCl, pH 8.0) + 24 ml buffer L2 (20 mM spermidine, 5 mM DTT, 100 mM ammonium sulfate, 5 mM EDTA). The cell suspension was transferred into Oak Ridge centrifuge tubes and lysed by sonication (Heat Systems Sonicator XL, Farmingdale, NY). Following ultracentrifugation (30,000 rpm, 30 minutes, 4°C), the supernatant was collected and protein precipitated with ammonium sulfate (380 g/L). The protein was pelleted by ultracentrifugation (30,000 rpm, 30 minutes, 4°C) and resuspended in 5 ml Buffer B (25 mM Hepes-KOH, pH 7.6, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol) and dialyzed in a Spectra/Por Membrane MWCO = 12,000 - 14,000 Da (Spectrum Medical Industries, Inc., Los Angeles, CA) against Buffer B (300 ml) two times. The sample was applied to a DEAE-Sepharose FF (Pharmacia) column (250 ml) and resolved by running a linear 50 mM - 550 mM KCl gradient over 500 ml in Buffer B. The fractions containing the highest amount of DnaK (as judged by SDS-PAGE) were pooled (50 ml), dialyzed against Buffer B (1000 ml) and applied to an ATP-agarose (Sigma, A2767) column (5 ml). The column was washed with 20 ml Buffer B + 500 mM NaCl followed by 10 ml Buffer B and eluted with 20 ml Buffer B + 5 mM ATP. The ATP-agarose eluent was applied to a MonoQ HR 5/5 column (Pharmacia), and free ATP was cleared from the column with Buffer B as judged by the return of the A_{280} trace to baseline. DnaK was resolved by running a linear gradient of 50 mM - 750 mM KCl over 30 ml in Buffer B. Fractions (1 ml) were collected, quick frozen on liquid nitrogen, and stored at -80°C. The DEAE-Sepharose FF and MonoQ columns were run on a Pharmacia FPLC system. All steps were carried out at 4°C. DnaK was judged to be >99% pure by the absence of any visible bands other than DnaK on a Coomassie stained SDS-PAGE gel.

ATPase Assays. Reaction mixtures (25 µl) contained ATPase Buffer (40 mM Hepes-KOH, pH 7.6, 50 mM KCl, 11 mM Mg(OAc)$_2$), [α-^{32}P]ATP or [γ-^{32}P]ATP, and DnaK. Radiolabeled ATP

stocks were made by adding radiolabeled ATP to unlabeled ATP to give a final activity of 82.6 μCi/ml. The total concentration of ATP was recalculated following the addition of the radiolabeled ATP. Unless otherwise indicated, the ATP and ATPase Buffer were mixed and preincubated at 30°C for 5 minutes, and hydrolysis was initiated by the addition of DnaK, marked as t=0 in the assays. When present in the reaction mixture, ADP and ovalbumin were preincubated with the ATP prior to the addition of DnaK. The reaction was incubated in a water bath at 30°C and was stopped at various times by spotting 2 ml of the reaction onto a PEI-cellulose TLC plate (J.T. Baker Inc., Phillipsburg, NJ). Spotting the mixture on the TLC plate stopped the reaction immediately, as reactions quenched with 1 N HCl prior to spotting and reactions spotted without chemical quenching show the same extent of hydrolysis. The TLC plate was developed in 1 M Formic Acid, 0.5 M LiCl, dried, and exposed to a Molecular Dynamics Storage Phosphor Screen. Data was obtained using a Molecular Dynamics PhosphorImager 445 Si and analyzed with ImageQuant 5.0. The amount each radiolabeled species present was determined by volume integration. The data was corrected for the level of background hydrolysis (typically 1% or less). The extent of hydrolysis was expressed as the fraction of ADP or P_i to total nucleotide, or further calculations were made to determine the velocity of the reaction.

ADP Kinase Assays. Reaction mixtures (25 μl) contained ATPase Buffer, [8-^{14}C]ADP, ATP, and DnaK. Radiolabeled ADP stocks were made by adding radiolabeled ADP to unlabeled ADP to give a final activity of 4.00 μCi/ml. The total concentration of ADP was recalculated following the addition of the radiolabeled ADP. The ATP, ADP, and ATPase Buffer were mixed and preincubated at 30°C for 5 minutes, and ADP phosphorylation was initiated by the addition of the DnaK sample, marked as t=0 in the assays. When CDP was used as a phosphate acceptor, the procedure used was exactly the same as above, except that [2-^{14}C]CDP was mixed with unlabeled CDP to make the radiolabeled CDP stock. The reaction was incubated in a water bath at 30°C and was stopped at various times by spotting 2 μl of the reaction onto a PEI-cellulose TLC plate. The TLC plate was developed and analyzed exactly as for the ATPase assays described above. The

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extent of ADP phosphorylation was expressed as the fraction of ATP to total nucleotide, or further calculations were made to determine the velocity of the reaction.

Rapid Gel Filtration Columns. Bio-Spin 30 columns (Bio-Rad, Hercules, CA) with an exclusion limit of about 40-kDa were used to remove free nucleotide from DnaK, allowing recovery of DnaK and nucleotide bound to DnaK. Columns were equilibrated in ATPase Buffer and separation was performed as described in the "Bio-Spin" manual. The Bio-Spin 30 columns efficiently removed all free nucleotide, as no detectable activity was present in the column flow through when [α-^{32}P]ATP alone was applied to the column.

DnaK Concentration Assays. The concentration of DnaK samples was determined using Bio-Rad Protein Assay Solution following the standard protocol. A DnaK sample that had a known concentration determined to precision by amino acid analysis (performed by the Biopolymers Laboratory at the M.I.T. Center for Cancer Research) was used as a standard in all DnaK concentration assays.

<center>**Results**</center>

Preparations of DnaK and Other Hsp70 Proteins Hydrolyze ATP with Biphasic Kinetics

In the experiments described below, we used DnaK that we had purified from DnaK overproducing *Escherichia coli* cells by a method that involves three primary chromatographic steps: DEAE-Sepharose anion exchange, ATP-agarose affinity, and MonoQ anion exchange columns. This method is basically the same used by other groups in the field [e.g. (29, 34, 56)]. Our preparations of DnaK were determined to be > 99% pure by SDS-PAGE analysis. ATPase assays using our preparations of DnaK showed that the rate of [α-^{32}P]ADP production appeared constant over time when the molar amount of [α-^{32}P]ATP in the assay was 100-fold or more greater than the molar amount of DnaK. However, when the molar amount of [α-^{32}P]ATP was less than 100-fold greater than the molar amount of DnaK, an initial burst of [α-^{32}P]ADP formation became apparent. When the amount of [α-^{32}P]ATP was only several fold greater than the amount of DnaK in the assay, the rate of [α-^{32}P]ADP formation clearly showed biphasic kinetics: an initial burst of ADP formation within the first minute, followed by a much slower steady-state rate of ADP formation. ATP was incubated with several different concentrations of DnaK at 30°C, and the amount of ADP produced was measured over time (Figure 1A). For our preparations of DnaK, the magnitude of the initial burst of ADP production increased directly with the amount of DnaK in the assay. From the results presented in Figure 1, the size of the initial bursts were determined by interpolation to be 1.39 μM, 2.87 μM, and 5.13 μM of ADP for 1.44 μM, 2.88 μM, and 5.77 μM of DnaK respectively. Thus, the molar size of the initial burst of ADP was approximately equal to the molar amount of DnaK present in the reaction, or 1 ADP: 1 DnaK. Since the size of the initial burst was stoichiometrically equal to the amount of DnaK, the burst was not detectable when the concentration of ATP approached or exceeded 100 times the concentration of DnaK, as the background amount of [α-^{32}P]ADP in the [α-^{32}P]ATP used in our assays was typically about 1% of the total nucleotide.

<center>40</center>

The possibility that the initial burst reflected the true steady-state rate of ATP hydrolysis by DnaK while the slower rate resulted from inactivation or loss of DnaK from solution was addressed. Previously, we had observed a significant decrease in the rate of ATP hydrolysis over time when DnaK was present at nanomolar concentrations. This loss of activity over time was determined to be due to the loss of DnaK from solution, and the addition of 2.0 μg/μl ovalbumin to the assay buffer to act as a non-specific carrier for DnaK corrected this problem. The addition of ovalbumin to assays with DnaK present at micromolar concentration had little effect on the kinetic profile of the ATPase reaction (Figure 1B), and therefore there was no significant loss of DnaK from solution at these concentrations. DnaK was also preincubated at 30°C for 30 minutes prior to the addition of ATP to determine if there was any thermal or time dependent loss of DnaK ATPase activity (Figure 1B). The kinetic profiles of DnaK ATPase with and without preincubation and with and without ovalbumin were all nearly identical, and thus demonstrated that the initial burst of ADP formation was a true characteristic of our DnaK preparations and was not due to any loss of enzymatic activity.

Several different preparations of DnaK from our lab were assayed, and all showed a stoichiometric burst of ADP production. Preparations of DnaK mutants T199S and T199A purified by the same procedure also showed an initial burst. In an effort to eliminate the possibility that the initial burst was due to some contaminant unique to our DnaK preparations, we obtained DnaK and other Hsp70 proteins from other labs and various commercial sources. Samples of DnaK from three major labs in the field, the 44-kDa amino-terminal ATPase fragment of DnaK from *Thermus thermophilus*, Hsp70 from one lab, and BiP from one lab all displayed similar initial burst activities in ATPase assays. Hsc70 purified from bovine brain purchased from a commercial source also showed an initial burst of ADP production. The observation of initial burst kinetics of ATP hydrolysis in all of these different preparations of Hsp70 proteins led us to conclude at the time that the burst activity was very likely to be an intrinsic property of the Hsp70 proteins themselves.

Majority of Nucleotide Bound to DnaK During Steady-State ATP Hydrolysis Is ATP

The presence of a stoichiometric initial burst of product formation is usually indicative of a reaction process in which the initial steps leading to product formation are faster than a rate-limiting step which follows product formation and is required for subsequent rounds of substrate turnover. Thus, the burst results from the first round of product formation, which would not be governed by the rate-limiting step. The following rounds must all pass through the rate-limiting step, resulting in the slower steady-state rate of product formation. Since all of the DnaK and Hsp70 samples we had tested behaved similarly, the observation of initial-burst kinetics in these preparations appeared to indicate that the binding and hydrolysis of the bond between the γ and β phosphates of ATP by Hsp70 proteins are fast steps and that some step following the hydrolysis of ATP must be rate-limiting in the ATPase cycle. This conclusion would have been significant since many researchers have concluded that ATP hydrolysis is the rate-limiting step in the ATPase cycle of DnaK and other Hsp70s (18, 21, 34, 44, 51).

We sought further confirmation that the observed burst of product formation was an intrinsic property of DnaK. If the assumption is made that ATP hydrolysis by DnaK is not reversible, then the majority of nucleotide bound to DnaK in steady-state hydrolysis should be ATP if hydrolysis is rate-limiting and ADP if some step following hydrolysis is rate-limiting. DnaK was therefore incubated with a saturating amount of ATP and allowed to reach steady-state. Free nucleotide was removed from DnaK by rapid gel filtration, and the nucleotide bound to DnaK was analyzed. Figure 2 shows that more ATP was bound to DnaK in steady-state hydrolysis than ADP, with 24.6% of the nucleotide as ADP and 75.4% of the nucleotide as ATP. This ratio of bound nucleotide was not consistent with non-reversible, fast hydrolysis of ATP. However, if ATP hydrolysis were reversible, then the ratio of ATP:ADP bound could have represented a rapid equilibrium between fast hydrolysis of ATP and fast rephosphorylation of ADP. See Chapter 5 of this thesis for a detailed discussion of these possibilities.

Biphasic Kinetics of ATP Hydrolysis by DnaK Preparations Is Not a Result of Product Inhibition

We considered the possibility that the initial burst reflected the true steady-state rate of ATP hydrolysis by DnaK while the slower rate resulted from product inhibition by the accumulating ADP. ADP has been shown to be a competitive inhibitor of DnaK ATPase activity (18, 37, 41). If the slower rate of hydrolysis resulted from inhibition by ADP, then the addition of ADP to the reaction mixture prior to the addition of DnaK should have reduced or eliminated the initial burst. Figure 3 shows that the addition of ADP to the reaction mixture actually resulted in an increase in the magnitude of the initial burst. ADP did cause a reduction in the slower steady-state rate of hydrolysis. Therefore, the biphasic kinetics of ATP hydrolysis by DnaK were not due to a sudden decrease in the rate of hydrolysis resulting from product inhibition.

We formulated a model to explain the surprising observation that the addition of ADP caused an increase in the magnitude of the initial burst together with the previously described observations. The initial burst kinetics and ratio of ATP to ADP bound to DnaK at steady-state seemed to indicate that ATP hydrolysis by DnaK is rapid and reversible. If the release of phosphate from DnaK were the rate-limiting step in the ATPase cycle and the fast, reversible release of ADP preceded phosphate release, then the presence of free ADP would allow DnaK to attach the free phosphate onto ADP, phosphorylating it to ATP. The DnaK could then release this ATP, bind a radiolabeled ATP, and quickly hydrolyze it, adding to the magnitude of the burst. Thus, each unlabeled ADP molecule would allow DnaK to recycle without proceeding through the slow step, and the DnaK could quickly hydrolyze another labeled ATP. This model is discussed in detail in Chapter 5 of this thesis.

DnaK Preparations Saturated with ATP Show a Reduced Initial Burst

If the DnaK ATPase activity were to follow initial burst kinetics, then saturating the DnaK with substrate prior to the ATPase assay would eliminate any initial burst. The reasoning behind this prediction was that the ATP loaded onto the DnaK would be quickly hydrolyzed, and the

DnaK would pause at the slow step in the ATPase cycle. DnaK was saturated with ATP, and the excess nucleotide was removed from the DnaK by rapid gel filtration. The DnaK was immediately mixed with radiolabeled ATP, incubated at 30°C, and the kinetics of the formation of ADP were measured (Figure 4). Presaturation of DnaK with ATP reduced the magnitude of the initial burst approximately six-fold, but did not eliminate it. We reasoned that some fraction of the DnaK could have completed the slow step of the ATPase cycle prior to being assayed and have produced a reduced initial burst of ADP production.

DnaK Preparations Phosphorylate ADP to ATP in the Presence of ATP

Based upon the model for the mechanism of DnaK ATPase activity outlined previously in this section, the following prediction was made. If radiolabeled ADP were added to a mixture of DnaK and unlabeled ATP, the DnaK would hydrolyze ATP and release ADP, entering into the "energized" DnaK*·P_i state, in which the energy from hydrolysis was either stored in a covalent bond between the phosphate and DnaK or in a conformational change of DnaK. Since ATP hydrolysis and ADP release would be reversible according to the model, radiolabeled ADP could bind to the DnaK*·P_i, become phosphorylated, and be released as radiolabeled ATP. Thus one would expect to see the production of radiolabeled ATP from the incubation of DnaK, unlabeled ATP, and radiolabeled ADP, if the model were correct. For a detailed discussion of this model and the basis for this prediction, see Chapter 5 of this thesis.

DnaK was therefore incubated with ATP and [8-^{14}C]ADP at 30°C, and the radiolabeled nucleotide content of the mixture was analyzed at various times. Figure 5A shows that [8-^{14}C]ATP was produced under these conditions. A quantitative kinetic analysis of the results (Figure 5B) shows that the amount of [8-^{14}C]ATP rapidly accumulated to the level that would be predicted for equal sharing of gamma-phosphates by the amounts of labeled and unlabeled adenosine nucleotide in the reaction mixture (see Chapter 5). The amount of [8-^{14}C]ATP then decreased at a slow, constant rate. This decrease in the amount of [8-^{14}C]ATP was presumably due to the steady-state hydrolysis of [8-^{14}C]ATP to [8-^{14}C]ADP by DnaK.

This ADP kinase activity that we found in our preparations of wild-type DnaK was also present in our preparations of the mutant DnaK T199S and T199A proteins. To address the possibility that the ADP kinase activity might have been due to some contaminant unique to the DnaK preparations from our lab, the same DnaK, Hsp70, Hsc70, and BiP preparations discussed earlier in this section were assayed. DnaK preparations from three different labs, the amino-terminal ATPase fragment of DnaK from *Thermus thermophilus*, a Hsp70 preparation, a BiP preparation, and Hsc70 purified from bovine brain were all found to possess an ADP kinase activity with kinetics similar to those of our DnaK preparations (data not shown).

DnaK Does Not Have a Detectable Phosphorylated Intermediate

The possibility that the phosphate in the putative intermediate "energized" DnaK*·Pi state might be covalently bound to DnaK (see Chapter 5 for a discussion) was addressed. We first looked for a phosphorylated intermediate of DnaK by incubating DnaK with $[\gamma\text{-}^{32}P]$ATP in ATPase Buffer, subjecting the sample to SDS-PAGE, and determining if the DnaK band was radiolabeled. There was no measurable activity in the DnaK bands following this procedure. We then considered the possibility that the putative phosphorylated intermediate of DnaK might be too short lived to be detected by this procedure. We therefore tried a different strategy for detecting a short-lived phosphorylated intermediate that consisted of incubating DnaK with $[\gamma\text{-}^{32}P]$ATP, denaturing the DnaK to remove non-covalently bound nucleotide and phosphate, and removing free nucleotide from the DnaK by rapid gel filtration. To test the effectiveness of various denaturants for removing bound nucleotide from DnaK, DnaK incubated with $[\alpha\text{-}^{32}P]$ATP was treated with urea, guanidine, and SDS. Since the alpha-phosphate of ATP is not predicted to covalently bind to DnaK, the successful removal of radioactivity from DnaK incubated with $[\alpha\text{-}^{32}P]$ATP should correlate directly with a denaturant's ability to remove non-covalently bound nucleotide from DnaK. DnaK was incubated with $[\alpha\text{-}^{32}P]$ATP and treated with urea, guanidine hydrochloride, or SDS and then incubated at 80°C or room temperature. The mixture was then applied to a rapid gel filtration column to remove free nucleotide. This entire procedure following

the incubation of DnaK with [γ-^{32}P]ATP took approximately 5 minutes. Table 1 shows that urea leaves a relatively high amount of ATP and ADP bound to DnaK, while guanidine hydrochloride is most effective at removing the nucleotide and does not require an incubation at 80°C to effectively remove most nucleotide. DnaK was incubated with [γ-^{32}P]ATP, denatured with urea or guanidine hydrochloride, and run over a gel filtration column. Table 1 shows that the fraction of DnaK with ATP or ADP bound following incubation with [α-^{32}P]ATP was 0.00298 when guanidine was used as a denaturant. The fraction of DnaK with ATP or P_i bound following incubation with [γ-^{32}P]ATP was 0.00275 when guanidine was used as a denaturant. Thus, the amount of radioactivity bound to DnaK when incubated with [γ-^{32}P]ATP and denatured was not significantly greater than the amount of radioactivity bound when incubated with [α-^{32}P]ATP and denatured, and therefore no evidence was obtained for a phosphorylated intermediate of DnaK.

ADP Kinase and Initial Burst Activities of DnaK/Hsp70 Preparations Are Due to the Presence of a Very Small Amount of Copurifying Protein

Despite the presence of the initial burst and ADP kinase activities in Hsp70 preparations from multiple sources, we became concerned that the activities present in the preparations which we had assayed were due to a small amount of some copurifying protein common to all of the preparations. No protein bands other than the 70-kDa band of DnaK were visible following Coomassie Brilliant Blue staining of a SDS-PAGE gel loaded with as much as 50 μg of DnaK per lane (data not shown). Since Coomassie staining allows the detection of as little as 0.1 μg of protein in a single band (45), our DnaK preparations were greater than 99% pure. It came to our attention, however, that in some cases the presence of very small amounts of the 16-kDa protein nucleoside diphosphate kinase (NDP kinase) in preparations of some proteins had resulted in a flawed analysis of the ATPase activity of the protein (Bernd Bukau, personal communication). NDP kinase catalyzes the transfer of gamma-phosphate from a nucleoside triphosphate to a nucleoside diphosphate with a turnover rate of approximately 1000 sec.$^{-1}$ under conditions similar to those used in our ATPase assays (36). This turnover rate is 600,000 - 6,000,000 times higher

than most published values of the turnover rate of the ATPase activity of DnaK, and therefore a relatively tiny amount of NDP kinase could significantly effect the kinetic properties of the ATPase activity of a preparation of DnaK. It became a concern that an amount of NDP kinase, undetectable by standard SDS-PAGE analysis, might have been present in all of the DnaK/Hsp70 preparations we had analyzed and could have been responsible for some of the activities that we had measured.

A phosphorylated intermediate of NDP kinase exists during the transfer of the gamma-phosphate from a nucleoside triphosphate to a nucleoside diphosphate. The phosphate is covalently bond to a conserved histidine residue of NDP kinase (1). We therefore thought that if NDP kinase were present at very low levels in our DnaK preparations, it might be detectable as a radiolabeled, phosphorylated intermediate, even if it were not detectable on a Coomassie stained gel. DnaK preparations were incubated at 30°C for 5 minutes with 0.1 - 10 molar equivalents of [γ-^{32}P]ATP with or without ADP. The samples were then denatured in SDS-PAGE loading buffer with or without boiling and analyzed by SDS-PAGE. This is essentially the same procedure previously used to detect the phosphorylated intermediate of NDP kinase (36). No radiolabeled bands were visible at 16-kDa or at any other molecular weight point on the gels, even after prolonged exposure of the gels to a phosphorimaging screen (data not shown). Following this experiment, there was still no direct evidence of the presence of NDP kinase in our DnaK preparations.

NDP kinase is substrate non-specific and catalyzes the transfer of phosphate from any nucleoside triphosphate to any nucleoside diphosphate with nearly equal efficiency (27). DnaK, on the other hand, while reported to hydrolyze GTP, CTP, and UTP to some extent, hydrolyzes ATP with the greatest efficiency (33) and ATP is believed to be required for proper DnaK function (33, 40). Therefore, the ability of a DnaK/Hsp70 preparation to transfer the gamma-phosphate from ATP and no other nucleoside triphosphate to ADP and no other nucleoside diphosphate would have provided compelling evidence that the DnaK/Hsp70 protein itself is responsible for the ADP kinase activity. On the other hand, the ability of a DnaK/Hsp70 preparation to transfer the gamma-phosphate from any nucleoside triphosphate to any nucleoside diphosphate with equal

efficiency would have increased the likelihood that the activity is due to some other protein. All DnaK and Hsc70 preparations discussed above were therefore incubated with ATP and either radiolabeled ADP or radiolabeled CDP. Figure 6A shows that when radiolabeled ADP was provided as a phosphate acceptor, radiolabeled ATP was produced, while when radiolabeled CDP was provided as a phosphate acceptor, radiolabeled CTP was produced with similar kinetics. Thus, the DnaK and Hsp70 preparations were non-specific as to which nucleoside diphosphate serves as a phosphate acceptor.

The presence of NDP kinase in a DnaK preparation would provide an alternative means for an initial burst of $[\alpha-^{32}P]ADP$ formation during the incubation of the preparation with $[\alpha-^{32}P]ATP$. Hsp70 proteins bind ADP with very high affinity (for Hsc70, $K_d = 1.8 \times 10^{-8}$ M), and ADP cannot be removed from Hsp70 by simple dialysis (19). Our preparations of DnaK as well as the DnaK and Hsp70 preparations from other labs described in this section were not treated to remove ADP and are assumed to have some ADP bound. Thus, NDP kinase would be able to use this Hsp70-bound ADP as a phosphate acceptor and rapidly transfer the gamma-phosphate from some of the ATP in the incubation mixture onto the ADP, at the same time converting the substrate ATP into ADP. If $[\alpha-^{32}P]ATP$ were used as a substrate, this phosphate transfer would create a burst of $[\alpha-^{32}P]ADP$ formation which would mimic the burst of $[\alpha-^{32}P]ADP$ formation expected from a single, rapid round of ATP hydrolysis. If, however, $[\gamma-^{32}P]ATP$ were used as a substrate, the transfer of radiolabeled gamma-phosphate to ADP would only reform $[\gamma-^{32}P]ATP$, indistinguishable from the original substrate, and there would be no initial burst of product formation. A single, rapid round of ATP hydrolysis, by contrast, would result in a initial burst of $^{32}P_i$ formation. To summarize: if the results shown in Figure 1 were due to a true initial burst of ATP hydrolysis by DnaK, then there should be an initial burst of $[\alpha-^{32}P]ADP$ formation when DnaK is incubated with $[\alpha-^{32}P]ATP$ and an initial burst of $^{32}P_i$ formation when DnaK is incubated with $[\gamma-^{32}P]ATP$. If, however, the results shown in Figure 1 were due to the rapid transfer of gamma-phosphate from ATP to ADP catalyzed by NDP kinase or some other protein, then there should be an initial burst of $[\alpha-^{32}P]ADP$ formation when DnaK is incubated with $[\alpha-^{32}P]ATP$ but

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no initial burst of $^{32}P_i$ formation when DnaK is incubated with [γ-^{32}P]ATP. The latter results could be explained within the context of the model for DnaK ATPase outlined in this section without evoking another protein as explained in Chapter 5. DnaK preparations were incubated with [α-^{32}P]ATP or [γ-^{32}P]ATP with or without ADP present in the incubation mixture. Figure 7 shows that when our DnaK preparations were incubated with [α-^{32}P]ATP, an initial burst of [α-^{32}P]ADP was present. The size of this burst of [α-^{32}P]ADP formation is increased when ADP is present in the incubation. However, when our DnaK preparations were incubated with [γ-^{32}P]ATP, no initial burst of $^{32}P_i$ was present, whether or not ADP was present in the incubation (Figure 7). Therefore, the initial burst of ADP production shown in Figure 1 resulted from the transfer of gamma-phosphate from [α-^{32}P]ATP to unlabeled ADP and not from the hydrolysis of [α-^{32}P]ATP. The size of the initial burst increased when ADP was added to the incubation mixture (Figure 3 and Figure 7), because more ADP molecules were available to serve as a phosphate acceptors. The size of the initial burst decreased when the DnaK was preincubated with ATP (Figure 4), because the ATP replaced the ADP bound by DnaK, resulting in the availability of fewer ADP molecules to serve as phosphate acceptors.

While the results shown in Figure 6 and Figure 7 provided evidence that the initial burst and ADP phosphorylation activities were not properties of DnaK, the results could still be fit into a model for the ATPase activity of DnaK as described in Chapter 5. Our first direct evidence that the ADP kinase activity was not a property of DnaK and other Hsp70s came from measurements of the biochemical activities of a preparation of DnaK from *Thermus thermophilus*. A preparation of the 44-kDa amino-terminal fragment of DnaK from the thermophilic bacterium *Thermus thermophilus* transgenically expressed in, and purified from, *Escherichia coli* cells was found to possess the previously described ADP kinase activity. If the ADP kinase activity were a property of the *T. thermophilus* DnaK, it would be expected to have an activity vs. temperature profile similar to the ATPase activity of the DnaK. If, however, the ADP kinase activity were a property of some copurifying protein from the *E. coli* cells, the temperature profiles of the ADP kinase and ATPase activities would be expected to be quite different. The ATPase and ADP kinase activities of the *T.*

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thermophilus DnaK 44-kDa N-terminal preparation were measured over a temperature range of 30°C to 90°C (Figure 8). While the ATPase activity increased steadily with temperature, the ADP kinase activity decreased with temperature increases over 30°C. The ADP kinase activity was abolished by 90°C, while the ATPase activity was very robust at this temperature. Therefore, these results indicated that the ADP kinase activity in the *T. thermophilus* DnaK preparation is a property of a copurifying *E. coli* protein, and strongly implied that the ADP kinase and initial burst activities in the DnaK and Hsp70 preparations discussed throughout this section are also almost certainly a property of a copurifying protein which is present at very low levels in the preparations.

Discussion

Preparations of *Escherichia coli* DnaK from our lab, which have been purified by a procedure highly similar to those employed by numerous other labs, were found to contain an ADP kinase activity that is not an intrinsic property of the DnaK itself. The protein which co-purifies with DnaK and is responsible for this activity is not unique to our preparations, as we detected the same kinase activity in *E. coli* DnaK samples from other labs, the 44-kDa amino-terminal ATPase domain of *Thermus thermophilus*, human Hsp70, bovine Hsc70, and hamster BiP. In all, we found ADP kinase activity in preparations from six different laboratories and one commercial source. This kinase activity in nucleotide nonspecific, as CDP is phosphorylated as readily as ADP. Nucleoside diphosphate kinase (NDP kinase) seems to be the most likely candidate protein responsible for this activity, especially in light of the reported physical interaction between Hsc70 and a 16-kDa nucleoside diphosphate kinase protein from eukaryotic cells (32). NDP kinase has a very high turnover rate of approximately 1000 sec.$^{-1}$ (36), so very little of the protein would be required to account for the level of kinase activity in the preparations which we assayed. The highest level of ADP kinase activity present in any of the samples of DnaK from our lab that we assayed was approximately 20,000 pmole ADP phosphorylated/min/µl sample. It would require only about 5 ng/µl of NDP kinase, just below the detection limit of a Coomassie stained SDS-PAGE gel, to result in this amount of ADP kinase activity. NDP kinase is able to use any NTP or dNTP as a phosphate donor substrate and any NDP or dNDP as a phosphate acceptor substrate. Its presence would therefore be consistent with the nonspecific nature with regard to phosphate acceptor substrate which we observed. Finally, NDP kinase is ubiquitous and highly conserved. *E. coli* NDP kinase and human NDP kinase are 43% identical in their amino acid sequences (22). NDP kinases from different organisms are also remarkably similar in their tertiary protein structures (57). It is therefore likely that NDP kinases from different organisms would behave similarly during various chromatographic separations due to their similar physical properties, just as highly conserved Hsp70 proteins from different organisms behave similarly during different

chromatographic separations. NDP kinase binds ATP with high affinity (36), and would therefore be likely to co-elute with DnaK from an ATP-agarose column. NDP kinase has been shown to elute from a MonoQ anion-exchange column at a lower KCl concentration than Hsp70 (27), but trailing of the NDP kinase peak into the Hsp70 peak could compromise the success of this separation. *E. coli* NDP kinase forms stable tetramers with a molecular weight of 66-kDa (1), and thus gel filtration chromatography would also be ineffective at separating NDP kinase from the 69.1-kDa DnaK. During the course of this study, it was reported that a 16-kDa NDP kinase protein co-purifies and co-immunoprecipitates with Hsc70 purified from eukaryotic cells (32). Such a physical interaction, if it existed, between *E. coli* DnaK and NDP kinase would make a complete separation of the two proteins, even based upon significant physical differences, difficult.

The implications of the presence of NDP kinase in DnaK preparations is significant. Many discrepancies regarding the characterization of the ATPase activity of DnaK and other Hsp70 proteins exist in the literature. NDP kinase, in addition to a gamma-phosphate transfer activity, has an ATPase activity with a velocity much faster than that of DnaK (36). The presence of differing amounts of undetected NDP kinase in DnaK preparations from different labs could explain, at least in part, the range of k_{cat} values reported for the ATPase activity of DnaK from 0.018 min.$^{-1}$ (42) to 0.13 min.$^{-1}$ (13, 46) to 1.0 min.$^{-1}$ (59). Several labs have also described the existence of differing ATPase rates between different preparations of DnaK within their own lab and different fractions within the same preparation (44), (Bernd Bukau, personal communication). NDP kinase could also be responsible for the report of initial burst kinetics by DnaK during ATPase assays (3), similar to our observations, and for the report of an ADP kinase activity by DnaK (27), also similar to our observations. The wide range of values for the peptide, DnaJ, and GrpE stimulated rates of DnaK ATPase could also be due, in part, to the presence of a very small amount of copurifying NDP kinase.

Our initial excitement elicited by the discovery of the initial burst and ADP kinase activities prior to similar reports by other labs (3, 27) and prior to our realization that these activities were not intrinsic properties of DnaK, led us to formulate a novel model to describe the ATPase cycle of

DnaK. This model, which is described in detail in Chapter 5 of this thesis, stated that the hydrolysis of the γ-β phosphate bond of ATP by DnaK was rapid and reversible. This rapid step led to the conformational change of DnaK which results in peptide release. The slow, rate-limiting step of the ATPase cycle was the conformational conversion of DnaK to the low energy, high peptide binding affinity state without the rephosphorylation of ADP. The DnaK could scan the peptide substrate by rapidly switching between the high affinity DnaK·ATP and low affinity DnaK*·ADP·P_i states. DnaJ would stimulate the rate-limiting DnaK*·P_i to DnaK·P_i conversion that would lock the peptide into place in the DnaK substrate binding site. This model was particularly appealing because it resolved the paradox that non-hydrolyzable analogs of ATP do not induce conformational change and peptide release by DnaK (33), but ATP hydrolysis is not required for peptide release by DnaK (40). According to our model, ATP hydrolysis was required for peptide release, but hydrolysis as measured by the formation of its products, ADP and P_i, was slow. The model was also consistent with the observation that ATP binds to DnaK in two steps, a rapid binding step followed by a slower, but still rapid conformational rearrangement that results in peptide release (51). According to our model, the establishment of the DnaK·ATP <-> DnaK*·ADP·P_i equilibrium was this second step of ATP binding.

Although our model was initially formulated to account for a kinase activity that we now believe is due to a contaminant, a slight modification of the model is attractive for explaining the coupling of the ATP hydrolysis cycle of DnaK to the peptide binding and release cycle. Specifically, we propose that ATP could bind DnaK to yield an intermediate DnaK*·ADP·P_i state in which the β-γ phosphate bond has been broken, but its energy is preserved in a conformational change of DnaK which results in the release of peptide by DnaK. This model differs from our original model in that the ADP bound by the DnaK* state is not exchangeable and must remain bound to DnaK as long as it is in the energized conformation. As is the case with our previous model, this model is attractive because it provides a physical model for the second step of ATP binding to DnaK (51) as well as a specific explanation as to why non-hydrolyzable analogs of ATP

do not elicit the conformational change of DnaK that results in peptide release (33) even though

they bind to Hsc70 in a manner which is very similar to the binding of ATP to Hsc70 (16).

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Table 1. DnaK Does Not Have a Phosphorylated Intermediate During Steady-State Hydrolysis of ATP

ATP Label	Chemical Denaturation	Thermal Denaturation	Fraction of DnaK with phosphate bound
$[\alpha\text{-}^{32}P]$	Urea	none	0.175
$[\alpha\text{-}^{32}P]$	Guanidine-HCl	none	0.00298
$[\alpha\text{-}^{32}P]$	SDS	none	0.0111
$[\alpha\text{-}^{32}P]$	Urea	80°C, 3 min.	0.00807
$[\alpha\text{-}^{32}P]$	Guanidine-HCl	80°C, 3 min.	0.00205
$[\alpha\text{-}^{32}P]$	SDS	80°C, 3 min.	0.0387
$[\gamma\text{-}^{32}P]$	Guanidine-HCl	none	0.00275

DnaK (final concentration: 1.21 μM) was incubated with either $[\alpha\text{-}^{32}P]$ATP or $[\gamma\text{-}^{32}P]$ATP (final concentration: 3.33 μM) in ATPase Buffer at 30°C for 5 min. Following this incubation, an equal volume of 8M Urea, 6M Guanidine-HCl, or 10% SDS was added as a denaturant. The sample was then incubated for 3 minutes either at room temperature or at 80°C. The sample was run through a BioRad BioSpin 30 column to remove free nucleotide, and the radioactivity in the eluent was quantitated by scintillation counting after the addition of 5 ml Hydrofluor. The molar amount of α or γ phosphate in the eluent was calculated and expressed in the table above (last column) as a fraction of the molar amount of DnaK in the incubation mixture.
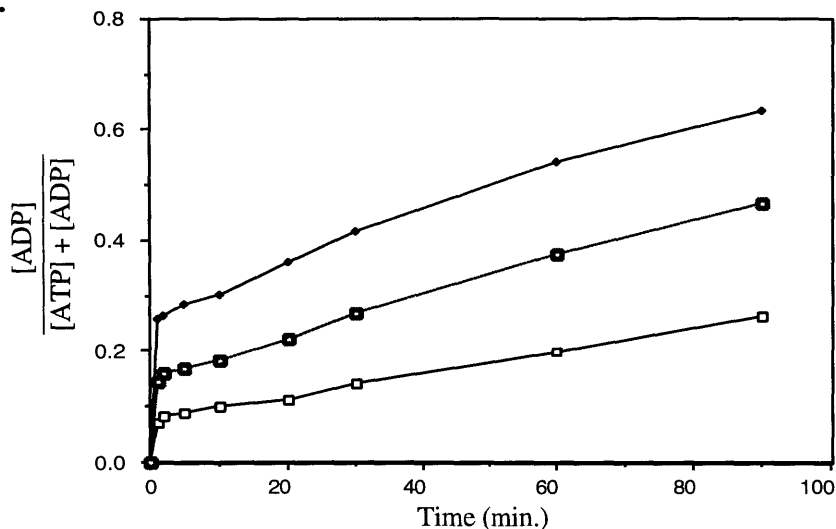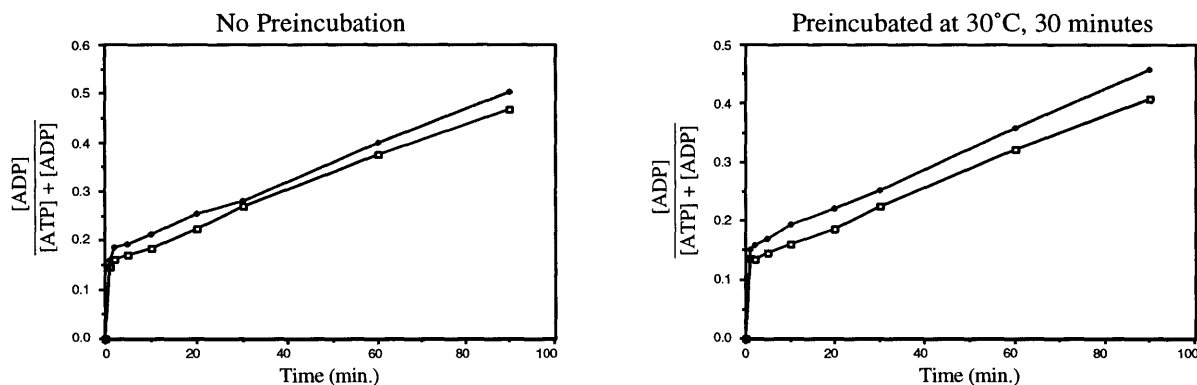
Figure 1. DnaK Preparations Show a Stoichiometric Initial Burst of ADP Production Which Preceeds Steady-State ATP Hydrolysis

(A) DnaK (final concentration: □, 1.44 µM ; ◘, 2.88 µM ; ♦, 5.77 µM) was added to [α-^{32}P]ATP (final concentration: 19.8 µM) in ATPase Buffer and incubated at 30°C for various times. Each incubation was spotted on a PEI-cellulose TLC plate, developed in 1 M Formic Acid, 0.5 M LiCl, and exposed to a phosphorimaging screen. The amount of ADP and total nucleotide in each reaction was determined by phosphorimaging analysis.

(B) DnaK (final concentration: 2.88 µM), either with no preincubation (left graph) or following a preincubation at 30°C for 30 min. (right graph) was added to [α-^{32}P]ATP (final concentration: 19.8 µM) in ATPase Buffer with (♦) or without (□) 2.0 µg/µl ovalbumin. The sample was incubated at 30°C for various times. Each incubation was spotted on a PEI-cellulose TLC plate, developed in 1 M Formic Acid, 0.5 M LiCl, and exposed to a phosphorimaging screen. The amount of ADP and total nucleotide in each reaction was determined by phosphorimaging analysis.

	%ATP	%ADP
Total Nucleotide	70.3	29.7
DnaK Bound Nucleotide	75.4	24.6
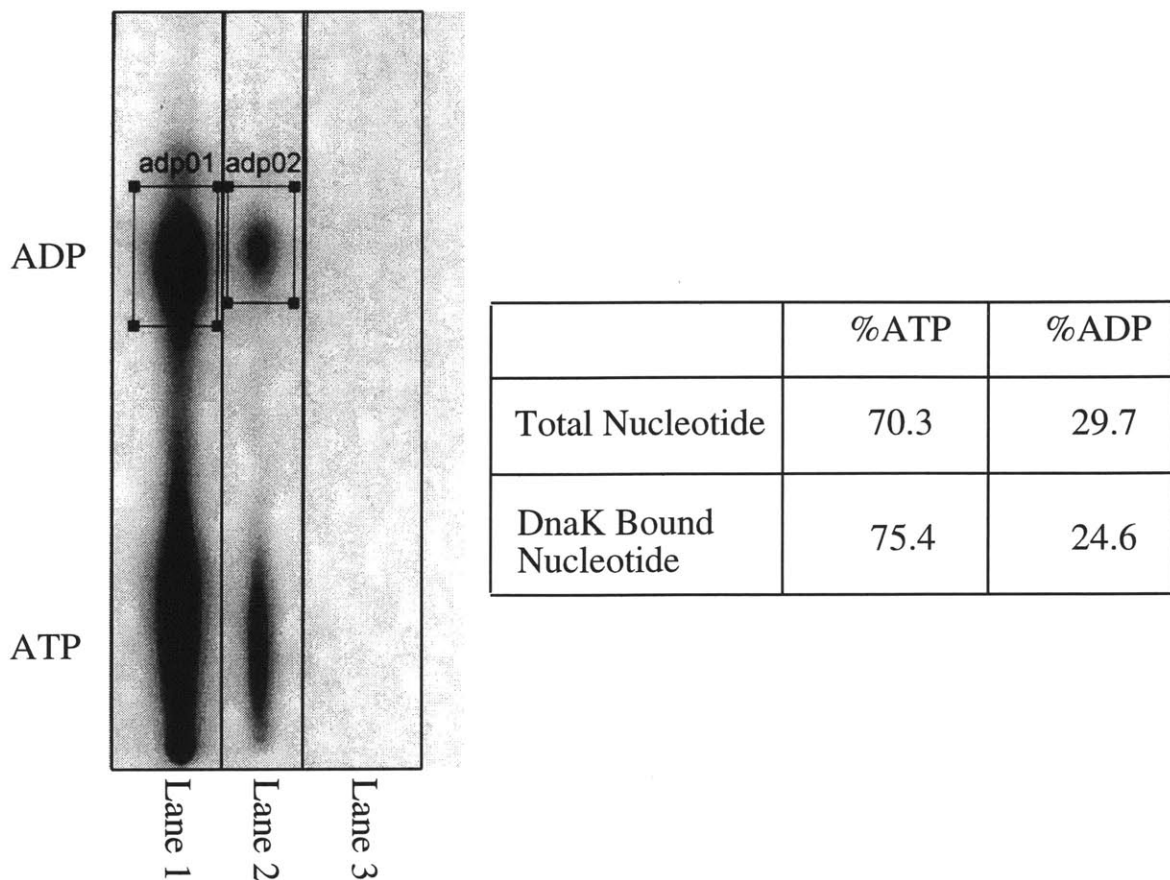
Figure 2. The Majority of Nucleotide Bound to DnaK During Steady-State Hydrolysis of ATP Is ATP

DnaK (final concentration: 5.77 μM) was added to [α-^{32}P]ATP (final concentration: 19.8 μM) in ATPase Buffer and incubated at 30°C for 5 minutes. Following incubation, the mixture was loaded onto a BioRad BioSpin 30 column to remove free nucleotide. Samples (2 μl) were spotted onto a PEI-cellulose TLC plate as follows:

Lane 1: DnaK + ATP, no spin column (DnaK bound nucleotide + free nucleotide)
Lane 2: DnaK + ATP, spin column as described above (DnaK bound nucleotide only)
Lane 3: ATP only, spin column (free nucleotide in spin column eluent)

The TLC plate was developed in 1 M Formic Acid, 0.5 M LiCl and exposed to a phosphor-imaging screen (exposure shown above). The amount of ADP and ATP was determined for each sample, and the results presented in the table above.
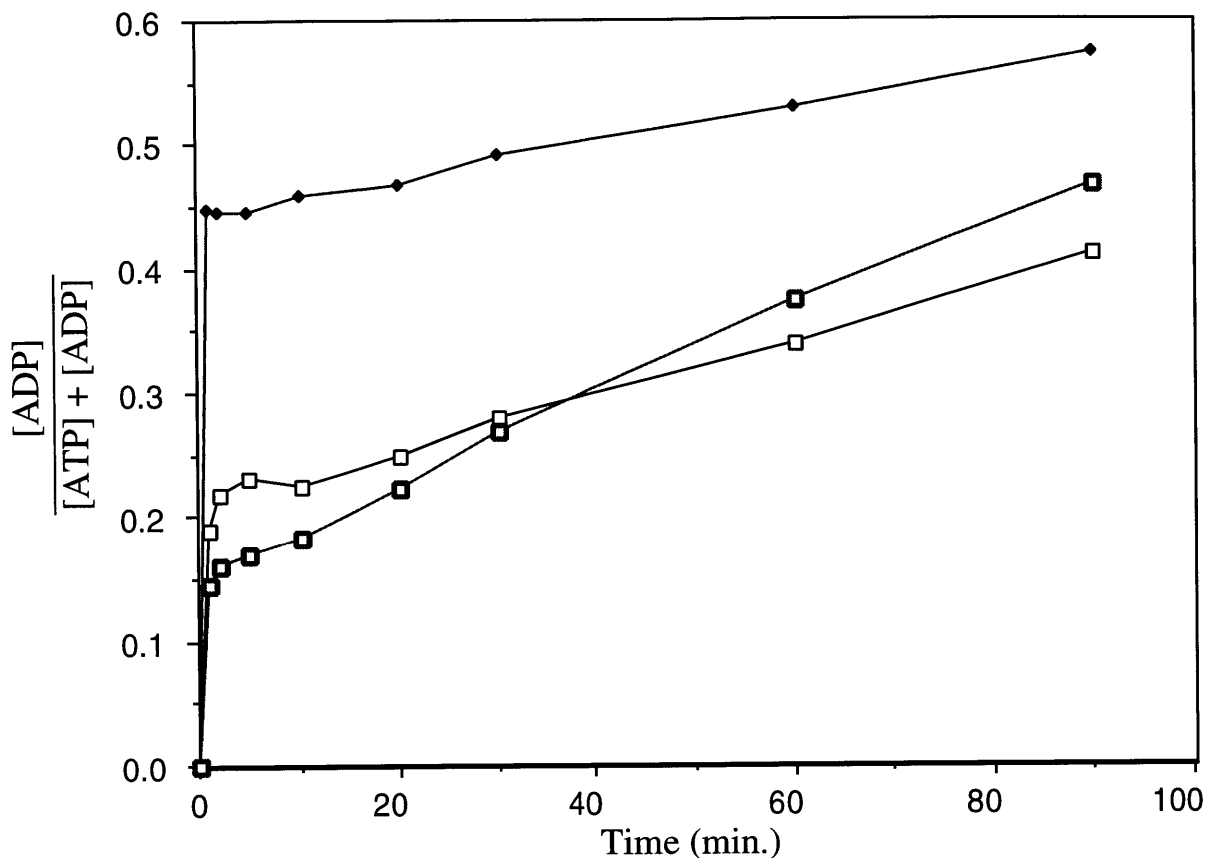
Figure 3. Biphasic Kinetics of ATP Hydrolysis by DnaK Preparations Is Not Due to Product Inhibition by ADP

DnaK (final concentration: 2.88 μM) was added to a mixture of [α-^{32}P]ATP (final concentration: 19.8 μM) and ADP (final concentration: □, 3.20 μM or ♦, 20.0 μM) or no ADP (■) in ATPase Buffer and incubated at 30°C for various times. Each incubation was spotted on a PEI-cellulose TLC plate, developed in 1 M Formic Acid, 0.5 M LiCl, and exposed to a phosphorimaging screen. The amount of ADP and total nucleotide in each reaction was determined by phosphorimaging analysis.
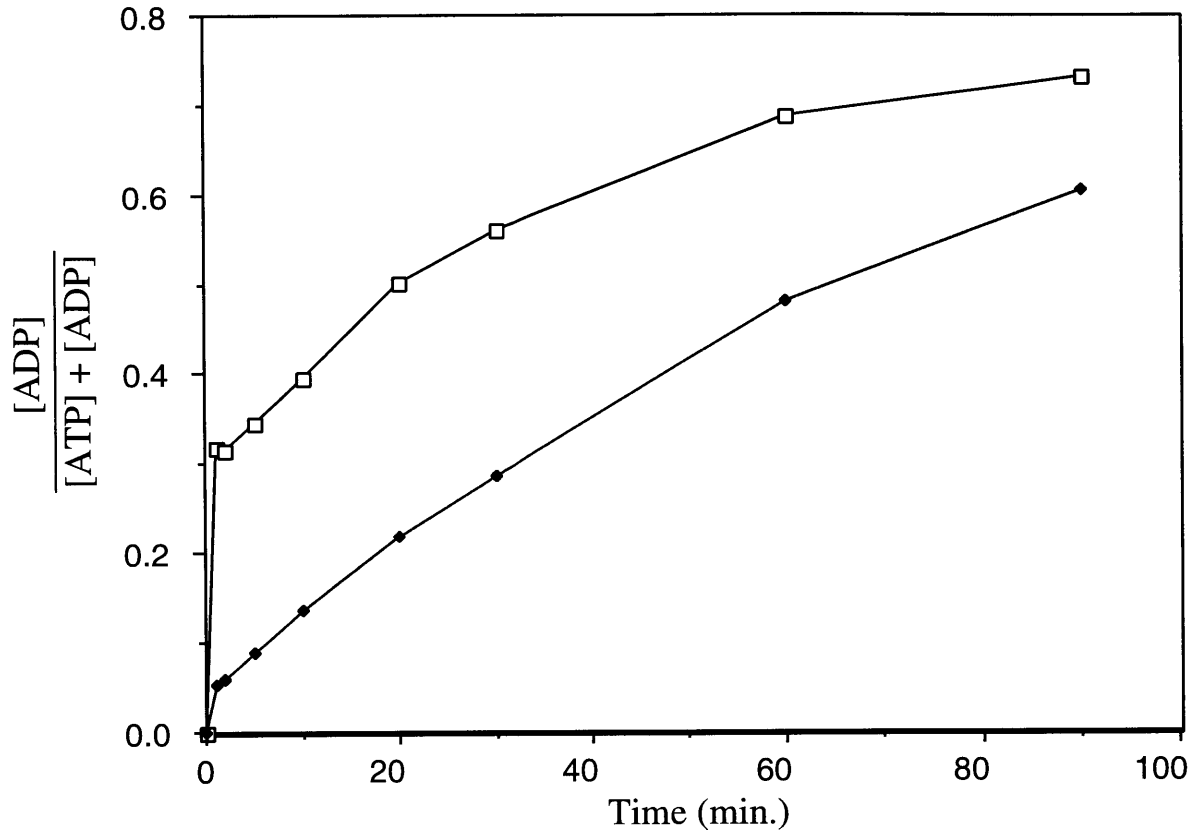
Figure 4. DnaK Preparations Saturated with ATP Show a Reduced Initial Burst

DnaK (3.01 μM) was incubated with a saturating concentration of ATP (20 μM) in ATPase Buffer at 30°C for 10 min. Free nucleotide was removed from the DnaK by running the sample through a Biorad BioSpin 30 column. DnaK saturated with ATP (●) (final concentration: 1.94 μM) or not saturated with ATP (□) (final concentration: 2.96 μM) was added to [α-^{32}P]ATP (final concentration: 19.8 μM) in ATPase Buffer and incubated at 30°C for various times. Each incubation was spotted on a PEI-cellulose TLC plate, developed in 1 M Formic Acid, 0.5 M LiCl, and exposed to a phosphorimaging screen. The amount of ADP and total nucleotide in each reaction was determined by phosphorimaging analysis.
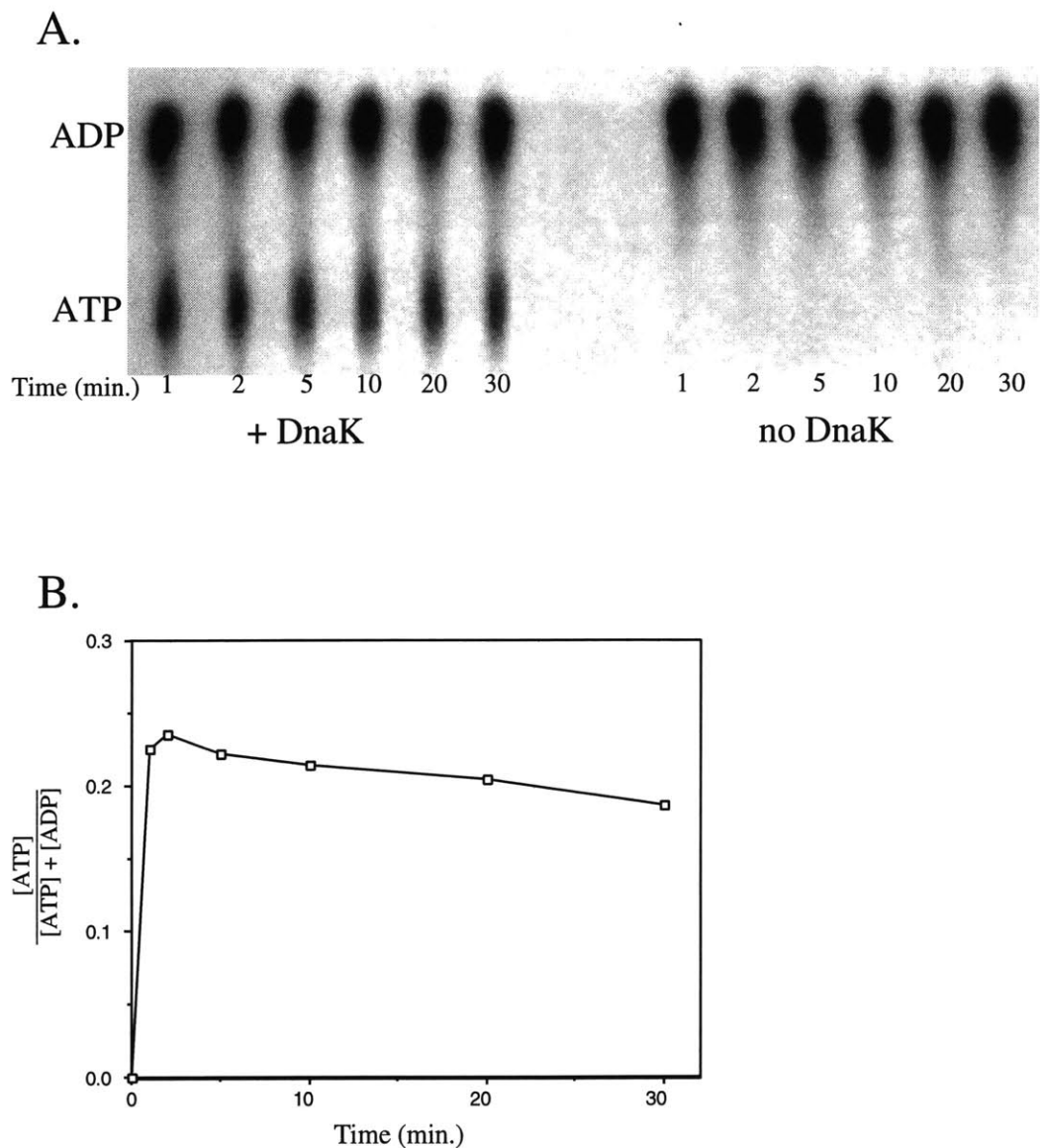
Figure 5. DnaK Preparations Phosphorylate ADP to ATP in the Presence of ATP

(**A**) DnaK (final concentration: 3.04 μM) was added to a mixture of ATP (final concentration: 20 μM) and [8-^{14}C]ADP (final concentration: 61.2 μM) in ATPase Buffer and incubated at 30°C for various times. As a control, [8-^{14}C]ADP and ATP were incubated with no DnaK. Each incubation was spotted on a PEI-cellulose TLC plate, developed in 1 M Formic Acid, 0.5 M LiCl, and exposed to a phosphorimaging screen.

(**B**) The amount of ATP and ADP for each time point was determined by phosphorimaging analysis and expressed as the ratio of ATP to total nucleotide.
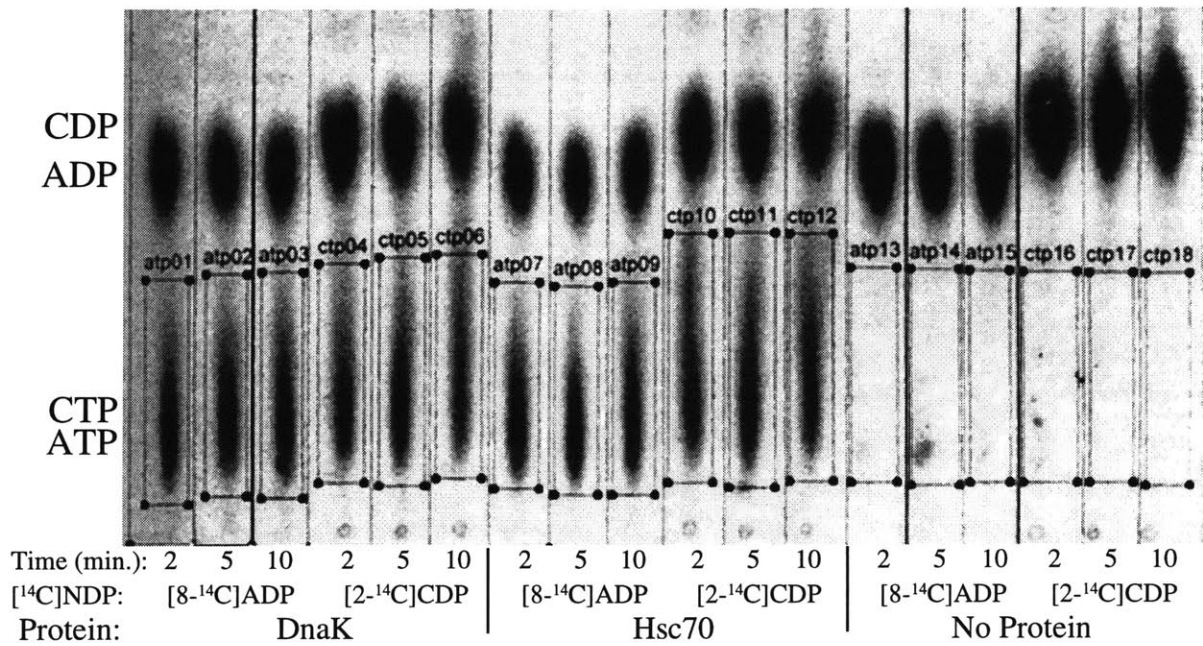
Figure 6. DnaK Preparations Have a Nonspecific Nucleotide Diphosphate Kinase Activity

DnaK (final concentration: 4.58 μM) or bovine Hsc70 (final concentration: 2.89 μM) was added to a mixture of ATP (final concentration: 40 μM) and [8-^{14}C]ADP (final concentration: 61.2 μM) or [2-^{14}C]CDP (final concentration: 59.2 μM) in ATPase Buffer. The samples were incubated at 30°C for various times. Each incubation was spotted on a PEI-cellulose TLC plate, developed in 1 M Formic Acid, 0.5 M LiCl, and exposed to a phosphorimaging screen.
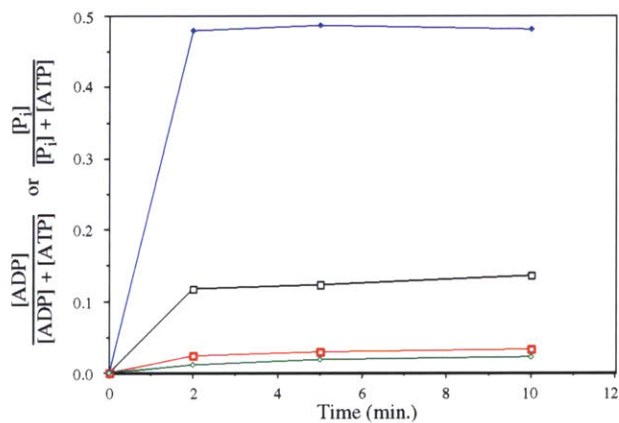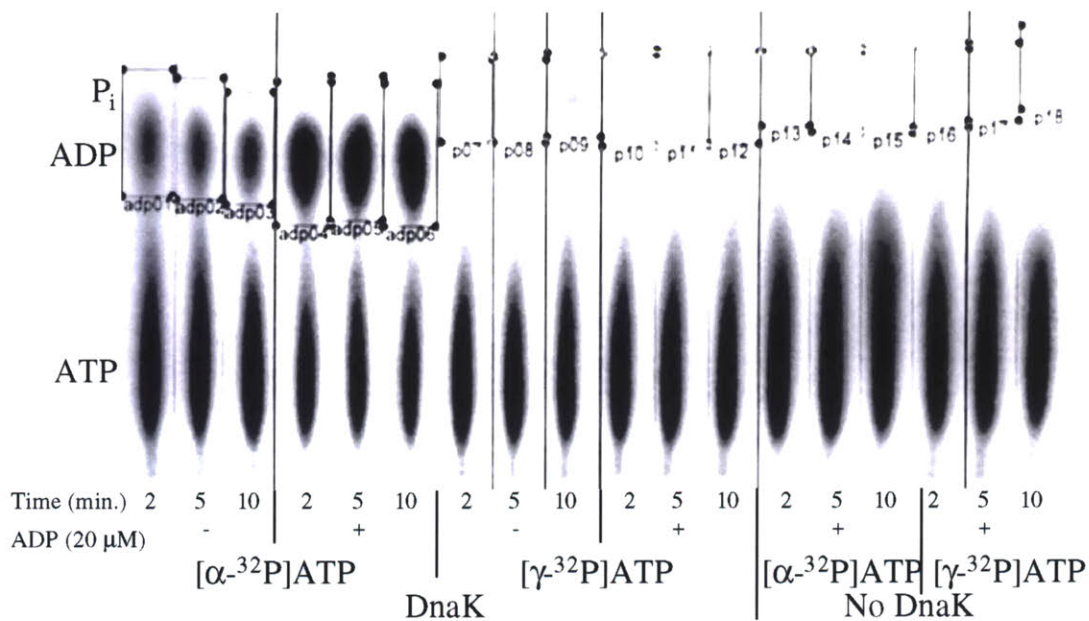
Figure 7. DnaK Preparations Show an Initial Burst of ADP Production, But Not an Initial Burst of Phosphate Production, When Incubated with ATP

DnaK (final concentration: 2.89 µM) was added to either [α-^{32}P]ATP or [γ-^{32}P]ATP (final concentration: 19.8 µM) and incubated at 30°C for various times. Some assays also contained ADP (final concentration: 20 µM). As controls, both [α-^{32}P]ATP and [γ-^{32}P]ATP were incubated without DnaK. Each incubation was spotted on a PEI-cellulose TLC plate, developed in 1 M Formic Acid, 0.5 M LiCl, and exposed to a phosphorimaging screen. The amount of ADP or P$_i$ and ATP were determined by phosphorimaging analysis and used to generate the graph above, showing DnaK incubated with: □, [α-^{32}P]ATP ; •, [α-^{32}P]ATP + ADP ; □, [γ-^{32}P]ATP ; ◦, [γ-^{32}P]ATP + ADP.

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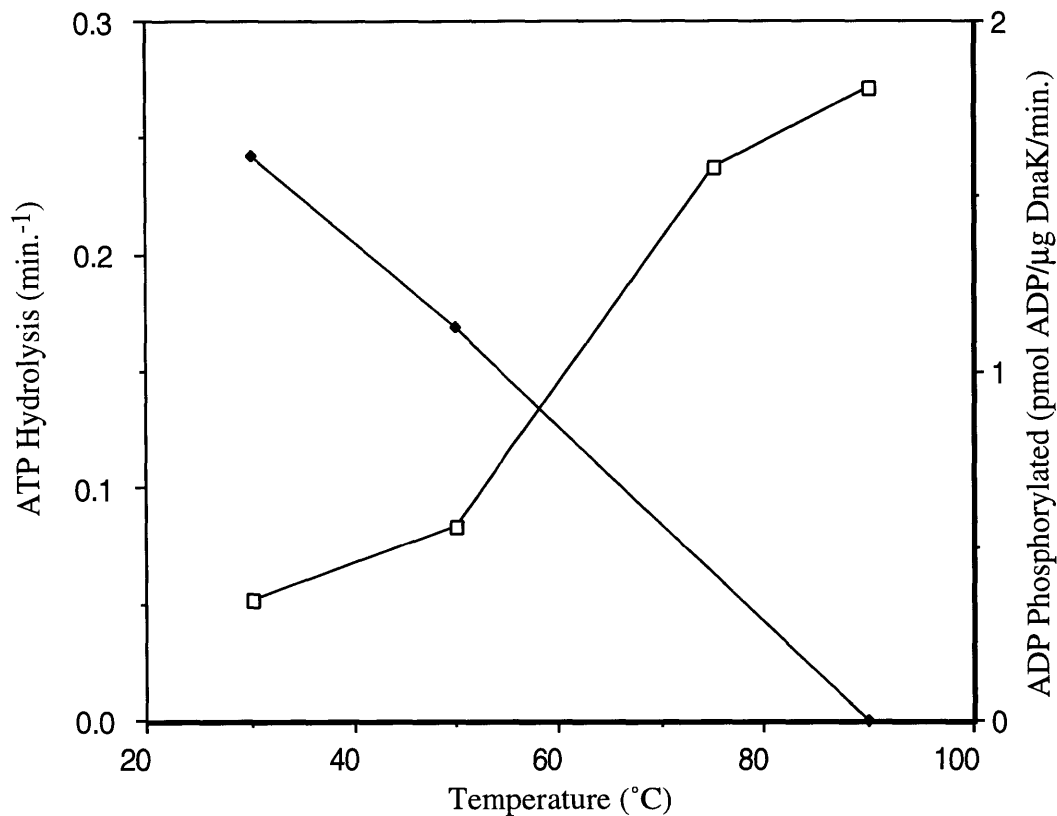
Figure 8. *Thermus thermophilus* DnaK 44-kDa N-terminal Domain Preparation ATPase and ADP Phosphorylation Activities Have Different Temperature Profiles

DnaK (44-kDa N-terminal domain) from *Thermus thermophilus* (final concentration: 4.56 µM) was added to [γ-^{32}P]ATP (final concentration: 40 µM) or [8-^{14}C]ADP (final concentration: 61.2 µM) in ATPase Buffer and incubated at various temperatures. The reactions were analyzed by the standard TLC separation and phorphorimaging quantitation. The rates of ATP hydrolysis (□) and ADP phosphorylation (◆) were determined at each temperature.

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Chapter 3

Analysis of DnaK Purified from *ndk::km Escherichia coli* cells

Abstract

The purification of DnaK from *E. coli* cells that carry a disrupted *ndk* gene, *ndk::km* , results in preparations with greatly reduced ADP kinase activities compared to preparations of DnaK purified from *ndk*+ cells. The reduction in the amount of ADP kinase activity in preparations of DnaK purified from *ndk::km* cells shows that nucleoside diphosphate kinase (NDP kinase) is responsible for most of the ADP kinase activity present in DnaK preparations. The remaining ADP kinase activity, which varies between preparations, is also a property of NDP kinase which is most likely expressed due to a low frequency reversion of the *ndk* gene. A weak, but measurable physical interaction exists between DnaK and NDP kinase and may be at least partially responsible for the co-purification of NDP kinase with DnaK. Multiple forms of DnaK exist during its purification which are revealed as multiple peaks during MonoQ chromatography. These multiple forms of DnaK do not represent an equilibrium between different conformational or multimeric states of DnaK. Although the different MonoQ peaks do not represent different nucleotide bound states of DnaK, the MonoQ chromatogram is simplified into one peak following treatment to remove bound nucleotide from DnaK. This nucleotide removal treatment also further reduces the amount of ADP kinase activity that co-purifies with DnaK.

Introduction

Escherichia coli, like all organisms, responds to sudden increases in temperature by transiently increasing the level of expression of a group of proteins called heat shock proteins. Over 20 genes belong to the heat shock regulon of *E. coli*, which consists of genes that are transcriptionally activated by the heat shock factor, σ^{32} (21). Three heat shock proteins of *E. coli*, DnaK, DnaJ, and GrpE, form a functional unit referred to as a chaperone machine (7). Together, these three proteins perform many roles in the cell, both at elevated and normal growth temperatures, that involve the folding and unfolding of proteins. They block the aggregation of some proteins and allow the reactivation of some heat-denatured proteins (26, 28). They also disassemble certain proteins complexes, including those required for the replication of λ (11) and P1 (33) phage. In addition, they repress the expression of heat shock genes at normal growth temperatures by negatively regulating the synthesis and stability of σ^{32} (3, 29).

DnaK plays the central role in the DnaK-DnaJ-GrpE chaperone machine. DnaK undergoes multiple cycles of binding and release of polypeptide substrate to facilitate the folding or prevent the aggregation of the polypeptide (4, 9). DnaK has a weak ATPase activity that is functionally linked to its peptide binding and release activity (15). DnaJ and GrpE act as cofactors by stimulating the ATPase activity of DnaK (14) and allowing it to perform its role as a molecular chaperone more efficiently .

Because of its importance in molecular chaperone funciton, the ATPase activity of DnaK has been the subject of intensive investigation. A true consensus description of the ATPase activity of DnaK, however, is lacking. A range of values have been reported for the k_{cat} of the ATPase activity (2, 22, 34), conclusions differ as to whether the ATP hydrolysis step of the ATPase cycle is fast (1) or rate-limiting (17, 24, 31), and conclusions differ as to whether ATP hydrolysis is reversible (10) or irreversible (17).

We previously found that preparations of DnaK and other Hsp70 proteins have ATPase activities which show initial burst kinetics and contain an ADP kinase activity. We concluded that

the initial burst and ADP kinase activities in these preparations are due to the presence of a very small amount of some copurifying protein. Although we were not able to obtain direct evidence that nucleoside diphosphate kinase (NDP kinase) was the copurifying protein, it seemed the most likely candidate. NDP kinase catalyzes the transfer of phosphate from nucleoside triphosphates to nucleoside diphosphates with a turnover rate of ~1000 sec.$^{-1}$. The presence of this protein, even in relatively tiny amounts, could explain the wide range of values reported for the rate of the ATPase activity of DnaK, as well as the initial burst (1) and ADP kinase (10) activities reported by other researchers.

We attempted to remove this copurifying activity completely from our DnaK preparations in order to allow us to make accurate measurements of the ATPase activity of DnaK and DnaK mutants. Purification of DnaK from *E. coli* cells containing a disruption of *ndk*, the gene encoding NDP kinase, removed most, but not all, of the ADP kinase activity in the DnaK preparations. We also looked for a possible physical interaction between DnaK and NDP kinase. In the course of this work, the nature of the multiple forms of DnaK which are revealed as multiple peaks of DnaK upon MonoQ anion-exchange chromatography was also investigated. We were able to convert DnaK into a form that chromatographed as a single peak rather than multiple peaks upon MonoQ chromatography, a finding which allowed for a better separation of DnaK from the ADP kinase activity.

Materials and Methods

Reagents and Media. Reagents and media were as described in Chapter 2 of this thesis. In addition, kanamycin and imidazole were purchased from Sigma, and adenylyl-imidodiphosphate (AMP-PNP) was purchased from Boehringer Mannheim. All restriction endonucleases and DNA ligase were purchased from New England Biolabs (Beverly, MA). Kanamycin was used at 50 μg/ml.

Construction of *ndk::km* Strains. The *Escherichia coli* strains NA7623 and JC7623 were generously provided to us by the laboratory of Masayori Inouye. Strain NA7623 has the gene encoding nucleoside diphosphate kinase (*ndk*) disrupted and is otherwise isogenic to JC7623. The *ndk* gene of NA7623 is disrupted by a kanamycin-resistance gene which is inserted into its EcoRI site as described (16). The *ΔdnaK52* allele was transduced into both NA7623 and JC7623 by using P1(GW8306) lysate as described (19). The resulting strains were TB3200 (*ndk::km ΔdnaK*) and TB3500 (*ndk+ ΔdnaK*) and were transformed with the DnaK overexpression plasmid pJM6 (18) by the standard CaCl$_2$/heat shock procedure (25) to create strains TB3220 (JC7623, *ndk::km ΔdnaK*, pJM6) and TB3520 (JC7623, *ndk+ ΔdnaK*, pJM6).

DnaK Purification. The standard protocol for the purification of DnaK was the same as described in Chapter 2 of this thesis. However, TB3520 was used to produce DnaK instead of GW8320, pJM6 and was grown in LB with ampicillin and chloramphenicol. TB3220 was used to produce DnaK in a *ndk::km* background and was grown in LB with ampicillin, kanamycin, and chloramphenicol.

Production and Purification of His-Tagged NDP Kinase. A plasmid was constructed to express NDP kinase with an amino-terminal tag of 10 histidine residues. Two 30mer primers were purchased from Gibco BRL designed to amplify the *ndk* gene from genomic *Escherichia coli* DNA

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based on the known sequence of the gene (8) and include a 5' NdeI and a 3' BamHI restriction site. The NdeI-*ndk*-BamHI product was amplified by PCR using the GeneAmp kit (Perkin Elmer Cetus, Norwalk, CT). The amplification product was purified by subjecting it to agarose gel electrophoresis and extracting its band using a QIAquick PCR purification kit (Qiagen). The purified product was digested with NdeI and BamHI and ligated into NdeI and BamHI linearized pET-16b plasmid (Novagen) to create the plasmid pET-16b/*ndk*.

The pET-16b/*ndk* plasmid was transformed into BL21(DE3)pLysS *E. coli* cells to create the strain TB2000. The TB2000 cells were grown in 800 ml of LB with ampicillin and chloramphenicol at 37°C to mid-log phase ($A_{600} \sim 0.6$) and induced with 0.4 mM IPTG to overexpress his-tagged NDP kinase (Ndk-N-his). Following incubation at 37°C for one hour, the cells were pelleted by centrifugation (5000 x g, 20 minutes, 4°C), resuspended in 1x binding buffer, low salt (5 mM imidazole, 50 mM NaCl, 20 mM Tris-HCl, pH 7.9), and lysed by sonication. Debris was removed from the lysed cells by ultracentrifugation (30000 rpm, 20 min., 4°C), and the supernatant was applied to a 2.5 ml His·Bind IMAC column (Novagen) equilibrated with 1x binding buffer, low salt. For co-purification experiments, the column was washed with 10 ml 1x binding buffer, low salt followed by 10 ml 0.5x wash buffer, low salt (30 mM imidazole, 50 mM NaCl, 20 mM Tris-HCl, pH 7.9). The gel bed was then resuspended in 0.5x wash buffer, low salt, and the gel suspension was transferred into a Pharmacia XK16 column. The XK16 column was attached to a Pharmacia FPLC system, and the gel bed was settled with 0.5x wash buffer, low salt at a flow rate of 0.5 ml/min. A linear gradient of 30 mM - 500 mM imidazole was then run over 30 ml in 0.5x wash buffer, low salt, and 1 ml fractions were collected. All purification steps were performed at 4°C.

Immunoblot Analyses. Fractions from the Ndk-N-his purification were collected, and a 10 μl aliquot of each sample was mixed with SDS-PAGE loading buffer (25), incubated in a boiling water bath for 3 minutes, and subjected to SDS-PAGE as described (25). The proteins were transferred from the gels to PVDF membranes, and antibody reactions and chemiluminescent

detection were performed using a "Western Lights" kit (Tropix, Bedford, MA) as described in the manual. The antibody used was affinity purified rabbit α-DnaK (1:5000 dilution).

ATPase Assays. Assays were performed as described in Chapter 2 of this thesis. The velocity of the ATPase reaction in terms of pmoles of ATP hydrolyzed/min. was determined by multiplying the measured $[P_i]/([ATP] + [P_i])$ ratio by the starting amount of ATP in the reaction (indicated in the corresponding figure for each reaction) and dividing by time.

ADP Kinase Assays. Assays were performed as described in Chapter 2 of this thesis. The velocity of the ADP kinase reaction in terms of pmoles ADP phosphorylated/min. was determined by multiplying the $[ATP]/([ATP] + [ADP])$ ratio by the starting amount of ADP in the reaction. The starting concentrations for each reaction were: $[ADP] = 61.2 \ \mu M$, $[ATP] = 400 \ \mu M$. When necessary, serial dilutions of the sample were made in ATPase Buffer in order to obtain a measurable activity.

Nucleotide Removal Treatment. Nucleotide was removed from DnaK by a method similar to that previously described for Hsc70 (6). DnaK was saturated with AMP-PNP (5 mM) in a total volume of 2 ml and incubated at 25°C for 1 hr. In order to remove free AMP-PNP, the sample was applied to a G-25M desalting column (Pharmacia PD-10 column) equilibrated with Buffer B and eluted with 3.5 ml Buffer B. The sample was dialyzed in a Spectra/Por Membrane MWCO = 12,000 - 14,000 Da against Buffer B (700 ml) three times. The dialyzed sample was applied to a MonoQ column and eluted with a double linear gradient of 50 mM - 225 mM KCl over 7.5 ml followed by 225 mM - 435 mM KCl over 45 ml in Buffer B.

DnaK Concentration Assays. The concentration of DnaK samples was determined as described in Chapter 2 of this thesis.

Protein Sequencing. Protein sequencing was performed at the M.I.T. Biopolymers Laboratory. The sample was loaded onto a Applied Biosystems PreSorb Sample Preparation Cartridge with a PVDF membrane. The sample was then sequenced with an Applied Biosystems Procise 494 Protein Sequencer.

<center>**Results**</center>

Purification of DnaK from *ndk::km E. coli* Cells Removes Most, But Not All, of the ADP Kinase Activity in DnaK Preparations

The observations presented in Chapter 2 that the kinase activity in DnaK and Hsp70 preparations does not show a substrate specificity limited to ADP, that the apparent initial burst of ADP production is due to the phosphorylation of ADP, and that the ADP kinase activity in *T. thermophilus* DnaK preparations is inactivated at high temperature lead to the conclusion that the ADP kinase activity is a property of some protein other than DnaK. The report, while this study was already in progress, that a 16-kDa protein identified as a member of the Nm23/nucleoside diphosphate kinase family co-purifies with Hsc70 (13) lent support to the hypothesis that the protein responsible for the ADP kinase activity in the DnaK and Hsp70 preparations that we analyzed is nucleoside diphosphate kinase (NDP kinase). To test this hypothesis, DnaK was purified from *E. coli* cells with a disruption in the *ndk* gene. A *ndk::km ΔdnaK* strain was constructed by transducing the *ΔdnaK52* allele into the *E. coli* strain NA7623. The *ndk* gene of NA7623 is disrupted by a kanamycin-resistance gene insert (16). The isogenic *ndk⁺ΔdnaK* strain was constructed by transducing the *ΔdnaK52* allele into the *ndk⁺* parent strain of NA7623, JC7623. The DnaK overexpressing plasmid pJM6 was then transfected into both the *ndk⁺ ΔdnaK* strain and the *ndk::km ΔdnaK* strain to make strains TB3520 and TB3220 respectively. DnaK was overexpressed in and purified from both strains following the standard purification protocol.

Fractions were collected from the final purification step, MonoQ anion-exchange chromatography, and assayed for ADP kinase activity and total protein concentration. Figure 1 shows overlaid chromatograms of the rate of ADP phosphorylation and the protein concentration of MonoQ fractions of DnaK purified from *ndk⁺* cells and *ndk::km* cells. In both cases, the protein and ADP kinase activity peaks overlap, although the ADP kinase activity reaches its highest level about 3 ml before the protein concentration reaches its highest level. Most significantly, while ADP kinase activity is present in both DnaK preparations, the amount of activity is

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approximately 500-fold lower in the *ndk::km* preparation than in the *ndk*+ preparation (Figure 1). Thus we concluded that most, but not all, of the ADP kinase activity in DnaK preparations was due to the presence of NDP kinase. It did not seem likely that the NDP kinase-independent ADP kinase activity was a property of DnaK, since the activity and protein peaks, while overlapping, do not coincide exactly (Figure 1). As is the case for DnaK purified from *ndk*+ cells, the only bands visible on Coomassie stained SDS-PAGE gels of the MonoQ fractions of DnaK purified from *ndk::km* cells are the 70-kDa bands of DnaK, even in the fractions with the highest ADP kinase activity (data not shown).

Attempts were made to separate the protein responsible for the remaining ADP kinase activity away from DnaK by adding extra chromatographic steps to the purification protocol. MonoQ purified DnaK was applied to a Superdex 75 gel filtration column. DnaK elutes from the Superdex 75 column in a single peak, but the ADP kinase activity co-elutes with the DnaK, and the Superdex 75 fractions containing the greatest amount of DnaK also contain the highest ADP kinase activities (data not shown). Heparin-agarose chromatography was also used in an attempt to separate the ADP kinase activity from DnaK, but DnaK and the protein responsible for the ADP kinase activity also co-elute from heparin-agarose columns (data not shown).

DnaK Has a Weak Physical Interaction with NDP Kinase

A recent publication (13) reports the co-purification of a 16-kDa NDP kinase protein with Hsc70 from eukaryotic cells. The authors also describe a physical interaction, co-immunoprecipitation, and functional interaction, stimulation of peptide-release by Hsc70, between the two proteins (13). We investigated whether or not a physical interaction also exists between *E. coli* DnaK and NDP kinase.

Co-purification by affinity chromatography was used as an assay to detect any physical interaction between the two proteins. The *ndk* gene was cloned by PCR and inserted into the plasmid pET-16b so that it would express NDP kinase with an amino-terminal tag of 10 histidine residues (Ndk-N-his). The pET-16b/*ndk* plasmid was transfected into BL21(DE3)pLysS *E. coli*

cells to create the strain TB2000. Proteins containing a stretch of histidine residues bind to Ni^{2+} ions and can be purified in a single step by IMAC (immobilized metal-ion affinity chromatography). TB2000 cells were induced to overexpress Ndk-N-his and lysed by sonication. The cell lysate supernatant was applied to a His-Bind IMAC nickel-column. The column was washed and eluted with a 30 mM - 500 mM imidazole gradient. Fractions (1 ml each) eluting from the column were collected. Samples of the TB2000 crude lysate supernatant, IMAC flow-through, IMAC wash, and the eluted fractions were run on SDS-PAGE and proteins were visualized by Coomassie staining (Figure 2). A large amount of Ndk-N-his protein, running at about 16-kDa on SDS-PAGE, is present in eluted fractions 8 - 23. Another protein, running at about 25-kDa, elutes at a slightly lower imidazole concentration than Ndk-N-his. This protein is likely the alpha subunit of succinyl-CoA synthetase which has been shown to physically interact with NDP kinase (12). A strong physical interaction between DnaK and Ndk-N-his would be indicated by the co-elution of DnaK with Ndk-N-his from the IMAC column. Careful inspection of the Coomassie stained SDS-PAGE gels in Figure 2 reveals very faint bands at about 70-kDa in fractions 12-16. SDS-PAGE gels identical to those shown in Figure 2 were run and blotted onto PVDF membranes for western blotting. The membranes were probed with α-DnaK antibody. The α-DnaK western blots are shown immediately below the respective Coomassie stained SDS-PAGE gels in Figure 2. The western blots show that a small amount of DnaK is present in the eluent fractions that contain the greatest amount of Ndk-N-his. However, only a small fraction of the DnaK present in the cells co-elutes with Ndk-N-his, as much larger amounts of DnaK appear in the column flow through and column wash lanes of the western blot (Figure 2). No DnaK, or any other protein, was detected in the eluent of a IMAC column loaded with the lysate of BL21(DE3)pLysS cells carrying only the vector pET-16b, showing that the DnaK does not interact with the IMAC column, and the DnaK co-elutes with Ndk-N-his bound to the IMAC column due to an interaction with Ndk-N-his itself. However, because of the small fraction of DnaK that binds to Ndk-N-his, the significance of this interaction is uncertain.

DnaK Elutes from MonoQ Anion-Exchange Columns in Multiple Peaks

The final step in the standard protocol for the purification of DnaK is MonoQ anion-exchange chromatography. Figure 3A shows the chromatogram of a typical MonoQ separation of DnaK purified from *ndk::km* cells. The chromatogram shows the trace of protein concentration as detected by its absorbance at 280 nm and the linear trace of the increasing KCl gradient. Figure 3A shows that DnaK elutes from the MonoQ column in at least several peaks. When the KCl gradient is made more shallow, a complex elution pattern of nine or more DnaK peaks emerges (Figure 3B). A similar MonoQ chromatogram is obtained for DnaK purified from *ndk*+ cells. Other groups have noted that DnaK elutes from MonoQ columns in multiple peaks and, furthermore, that the ATPase activity of the DnaK varies from peak to peak (17, 24). Neither the reason for the complex separation of DnaK by MonoQ nor the cause of the differing ATPase activities in different peaks has been determined. The researchers who have reported the multiple peaks have assumed that the peak with the lowest ATPase activity has unstimulated ATPase activity and used the DnaK from that peak in biochemical assays (17, 24).

We were concerned by the complex MonoQ separation of DnaK and particularly concerned by the varying ATPase activities between different peaks. In an effort to better understand the nature of the different peaks of DnaK, a more detailed biochemical analysis was made of each MonoQ fraction. ATPase assays were performed on each DnaK containing MonoQ fraction from the separation shown in Figure 3A. In order to avoid the appearance of "mock initial bursts" of [α-^{32}P]ADP formation due to the phosphorylation of ADP (Chapter 2), [γ-^{32}P]ATP was used as a substrate in the ATPase assays. The kinetics of formation of ^{32}P$_i$ for DnaK MonoQ fractions 6 - 17 are shown in Figure 4A. The kinetic profiles in Figure 4A show that, in addition to variations in the overall extent of ATP hydrolysis between fractions, the shape of the profile differs significantly between certain fractions. For example, fractions 7 and 8 show a large burst of P$_i$ formation within the first five minutes of hydrolysis followed by a much slower steady-state rate of P$_i$ production. The size of the initial bursts shows that about 45% of the total ATP is hydrolyzed to ADP + P$_i$ for both fractions within the first five minutes. To emphasize, since [γ-^{32}P]ATP was

used as a substrate, these bursts are due to true ATP hydrolysis resulting in the formation of free P_i and are not due to the phosphorylation of ADP. In contrast to fractions 7 and 8, fractions 12 - 17 have nearly linear kinetic plots with no initial burst. Fractions 9, 10, and 11, which contain the highest amounts of DnaK (see Figure 4B), do not show a pronounced initial burst, but do have a more rapid initial phase of ATP hydrolysis followed by a slower phase. The rates of ATP hydrolysis for each fraction were determined to characterize both the initial burst or fast initial phase, called the ATPase "burst" activity, and the steady-state rate of hydrolysis, called the ATPase "steady-state" activity. The ATPase "burst" rate was calculated by determining the rate of hydrolysis over only the first five minutes of the assay's incubation. The ATPase "steady-state" rate was calculated by determining the rate of hydrolysis between minutes 5 and 30 of the assay's incubation. In fractions with no initial burst or rapid initial phase, the "burst" rate and the "steady-state" rate are nearly equal. These values, along with the ADP phosphorylation rate and protein concentration for each fraction are shown in Figure 4B. The three activities, ADP kinase, ATPase "burst", and ATPase "steady-state", are plotted on separate graphs together with the protein concentration to show the MonoQ chromatograms of each activity together with the chromatogram of DnaK protein (Figure 4C). The highest amount of ADP kinase and ATPase "burst" activities elute from the MonoQ column prior to the highest amount of DnaK. Furthermore, the ADP kinase activity and ATPase "burst" activity elute primarily in the same fractions, and their chromatograms have very similar shapes. The ATPase "steady-state" activity, by contrast, elutes primarily in the same fractions as the majority of the DnaK, and their chromatograms closely coincide. These chromatograms indicate that the same protein, with an elution peak about 3 ml before the DnaK elution peak, is responsible for both the ADP kinase and ATPase "burst" activities. The ATPase "steady-state" activity is primarily associated with DnaK. However, measurable amounts of ADP kinase, and hence the protein responsible for it, are present in all DnaK containing fractions. Since the ATPase activity associated with this protein makes an unknown contribution to the ATPase activity in the DnaK containing fractions, reliable values of the ATPase activity of DnaK cannot be determined with this protein present in significant amounts.

Nature of the Multiple MonoQ Peaks of DnaK

MonoQ anion-exchange columns separate molecules based upon their charge. The different DnaK peaks therefore differ in their exposed surface charge. Three explanations as to the nature of the multiple peaks of DnaK which elute from MonoQ columns are:

1) The different peaks represent different states of DnaK in equilibrium with each other, for example different conformational or multimeric states in equilibrium.

2) The different peaks represent different states of DnaK along a time dependent pathway, for example different nucleotide-bound states (DnaK·ATP -> DnaK·ADP·P$_i$ -> DnaK·ADP -> DnaK).

3) The different peaks represent different states of DnaK that are static, for example states with different covalent modifications or having short truncations at the amino-terminal and/or carboxyl-terminal ends.

The strategy to distinguish between these possibilities was to collect fractions of DnaK eluting at different points (early and late) from a MonoQ column and to then individually reinject each fraction onto the MonoQ column and analyze the chromatogram. The predicted results for each of the three possibilities described above are:

1) If the different peaks represent different DnaK states at equilibrium, then reinjecting one peak should result in a chromatogram containing all of the original peaks.

2) If the different peaks represent different DnaK states along a time dependent pathway, then reinjecting one peak should result in a chromatogram containing that peak and possibly the peaks of the DnaK states downstream from it. Reinjecting the "earliest" form DnaK peak should result in a chromatogram possibly containing all peaks, while reinjecting the "latest" form DnaK peak should result in a chromatogram containing only that peak.

3) If the different peaks represent different DnaK states that are static, then reinjecting one peak should result in a chromatogram containing only that peak.

DnaK was purified from *ndk::km* cells according to the standard purification protocol. MonoQ fractions of DnaK from the final purification step were collected and individually reinjected onto

the MonoQ column. Figure 5 shows the MonoQ chromatograms of the original purification (blue) and of reinjected early (red) and late (green) eluting fractions superimposed for comparison. When individual fractions are reinjected onto the MonoQ column, the peak associated with that fraction is the predominant peak on the resulting chromatogram and one or two smaller peaks are also present. This pattern indicates that the different peaks of DnaK are not the result of an equilibrium phenomenon, since the reinjection of one peak does not result in a chromatogram containing all peaks. It is possible that the multiple peaks of DnaK result from different states of DnaK along a time dependent pathway. For example, reinjection of the late fraction results in a major peak and a smaller peak which elutes at the same position as the major peak resulting from the reinjection of the early fraction (Figure 5C). The chromatogram of the reinjected early fraction, however, has no minor peak the elutes at the same position as the major peak from the reinjected late fraction (Figure 5C). This pattern could indicate that the major peak of the late eluting fraction represents a state of DnaK that occurs earlier in a pathway than the state of DnaK represented by the major peak of the early eluting fraction. It is also possible that the peaks represent static states of DnaK, and the minor peaks in each reinjected chromatogram arise from shoulders of neighboring peaks which were included in the reinjected fraction. The shoulders of these neighboring peaks would elute at the center position for that peak following reinjection, creating resolution between the major and minor peaks.

Since the multiple MonoQ peaks of DnaK do not represent an equilibrium between different DnaK states, we investigated the possibility that the different peaks represent different static states of DnaK. One possibility considered was that the different states are different phosphorylated forms of DnaK. The reasons for our interest in this possibility were: 1) phosphorylation of a protein effects its charge and elution behavior from ion-exchange columns, 2) *in vivo* phosphorylation of DnaK has been observed and effects the peptide binding behavior of DnaK (27), 3) NDP kinase has been shown to phosphorylate proteins (32), and 4) a eukaryotic NDP kinase has been shown to functionally interact with Hsc70 by effecting its peptide binding behavior (13). If the different peaks represent different phosphorylated states of DnaK, then removal of the

phosphates should cause the multiple peaks to collapse into a single peak. All phosphoamino acids except for phosphotyrosine and phosphocysteine are acid and/or base labile (5), and proteins containing certain phosphorylated residues including phosphotyrosine can be dephosphorylated by treatment with alkaline phosphatase (30). Both acid and base treatment proved to be too harsh to allow an analytical MonoQ separation of the treated DnaK, and treatment of pooled DnaK MonoQ fractions with alkaline phosphatase resulted in no change in the MonoQ chromatogram (data not shown). DnaK samples from various MonoQ fractions were also analyzed by electrospray mass spectrometry in order to detect phosphorylation or other covalent modifications of DnaK or short truncations at the amino-terminal or carboxyl-terminal ends. However, the mass determinations of all samples was the same, within error, with a value of 69,136 Da (data not shown). Thus, neither covalent modifications nor truncations are responsible for the multiple peaks of DnaK resulting from MonoQ separation.

Treatment To Remove Nucleotide Results in One Predominant Form of DnaK

Pooled MonoQ fractions of DnaK (from the MonoQ separation shown in Figure 6A) were treated to remove bound ADP by saturating the DnaK with nonhydrolyzable AMP-PNP followed by extensive dialysis as previously described (6). When the nucleotide-free DnaK was reinjected onto a MonoQ column, it eluted as one major peak followed by several much smaller broad peaks (Figure 6B). The elution position of the peak in Figure 6B is the same as the earliest eluting peak in Figure 3B. In order to determine if the presence of nucleotide itself results in the multiple forms of DnaK which lead to the multiple peaks during MonoQ separation, the nucleotide-free DnaK peaks were collected from the MonoQ separation shown in Figure 6B and saturated with 2 mM ATP. Following a 15 minute incubation at 25°C, the DnaK was immediately loaded onto a MonoQ column. Figure 6C shows the MonoQ chromatogram of the nucleotide-free DnaK following saturation with ATP. The DnaK does not separate into multiple peaks following saturation with ATP, indicating that the presence of different nucleotide-bound states of DnaK is not the cause of the multiple peaks of DnaK on a MonoQ column. Some other change occurs to the DnaK during

the nucleotide removal treatment to convert the DnaK from several forms into one predominant form. One possible explanation for this conversion is that the treatment removes peptide fragments which are bound by DnaK.

NDP Kinase Activity Is Lowered in the Nucleotide-Free DnaK

Assays were performed on MonoQ fractions of the nucleotide-free DnaK in the same manner as described for the assays in Figure 4. Figure 7A shows the kinetic profiles of $^{32}P_i$ production for each fraction incubated with [γ-^{32}P]ATP. Fraction 13, which contains very little DnaK, has the most active ATPase activity with a rate that slows over time, much like fraction 9 in Figure 4. The ATPase activity in fractions 14 - 17 is much lower and nearly linear (Figure 7A). Figure 7B shows MonoQ chromatograms of the ATPase "burst", ATPase "steady-state", and ADP kinase activities overlaid with the chromatogram of the protein concentration. The ADP kinase and ATPase "burst" activities are present and elute at peak amounts about 2 ml prior to the peak of DnaK concentration. The presence of these activities indicates that the protein responsible for them is still present in the DnaK MonoQ fractions. However, the absolute level of the ADP kinase activity that co-elutes with the single peak, nucleotide-free DnaK is significantly lower than the ADP kinase activity that co-elutes with the multiple peak, untreated DnaK. The ADP kinase activity in MonoQ fraction 15, which contains the highest amount of nucleotide-free DnaK, is less than 0.2 pmole ADP phosphorylated/min/5 μl, nearly 50-fold lower than the 8.98 pmole ADP phosphorylated/min/5 μl for the MonoQ fraction containing the highest amount of untreated DnaK (Figure 4B).

The Protein Responsible for the ADP Kinase Activity In DnaK Preparations Purified from *ndk::km* Cells Is NDP Kinase

We noticed that some preparations of DnaK purified from *ndk::km* cells had an ADP kinase activity much higher than most of the other *ndk::km* DnaK preparations. The level of ADP kinase activity in the MonoQ fraction with the highest activity from the preparation with the highest

activity was 80,000 pmole ADP phosphorylated/min/5 µl sample, or nearly as high as the level of activity in the MonoQ fraction with the highest level of activity of DnaK purified from ndk^+ cells. Following the treatment to remove nucleotide and MonoQ chromatography, the highest level of ADP kinase activity in this sample was resolved from DnaK. The fraction with the highest amount of ADP kinase activity, MonoQ fraction 13, phosphorylated ADP at a rate of 15,000 pmole/min./5 µl sample. A sample of this MonoQ fraction (10 µl) was subjected to SDS-PAGE, and the silver stained gel revealed a single band of approximately 15-kDa. This sample was subjected to 10 rounds of amino-terminal peptide sequencing. A search of the *Escherichia coli* genome database revealed that the only DNA sequence in *E. coli* matching the protein sequence obtained (AIERTFSIIK) is the sequence spanning codons 2 to 11 of the *ndk* gene. Therefore, the protein responsible for the residual ADP kinase activity in DnaK preparations purified from *ndk::km E. coli* cells is NDP kinase. The concentration of NDP kinase in MonoQ fraction 13 was determined by the protein sequencing analysis to be 0.1 µM or 1.6 ng/µl. Assuming that the relationship between ADP kinase activity and the amount of NDP kinase protein is linear, the amount of NDP kinase in nucleotide-free, single MonoQ peak DnaK fractions with low ADP kinase activity (0.05 pmole ADP phosphorylated/min/µl sample) is approximately 0.03 pg/µl. The kanamycin gene disruption of the *ndk* gene was created by inserting a EcoRI fragment into the single EcoRI site of the *ndk* gene and did not include the removal of any segment of the *ndk* gene (16). Assuming the NDP kinase was produced in the *ndk::km* cells themselves, some sort of genetic rearrangement must have occurred during the incubation of the cells that resulted in the restoration of the *ndk* gene. Apparently, this event was random, and resulted in the production of vastly different amounts of NDP kinase between various incubations.

Discussion

DnaK purified from *E. coli* cells containing a disruption of the *ndk* gene typically results in approximately a 500-fold reduction in the level of co-purifying ADP kinase activity when compared to DnaK purified from *ndk*+ cells. This observation confirms our previous hypothesis (Chapter 2 of this thesis) that the ADP kinase activity found in preparations of DnaK is due to the presence of a very small amount of co-purifying NDP kinase. The identity of the protein responsible for the remaining ADP kinase activity in preparations of DnaK purified from *ndk::km* cells was initially unknown to us, as we had assumed that the kanamycin gene insertion in the *ndk* gene resulted in a complete knock-out of NDP kinase production. However, the presence of abnormally high levels of ADP kinase activity in some *ndk::km* preparations together with our ability to effectively resolve the ADP kinase activity from DnaK following the treatment of DnaK to remove nucleotide, allowed us to collect enough pure protein to allow amino-terminal protein sequencing. Surprisingly, the protein responsible for the residual ADP kinase activity was found to be NDP kinase. Southern blot analysis of the NA7623 strain which contains the *ndk::km* disruption showed that the *ndk* gene was effectively disrupted and no duplication of the *ndk* gene was present in the strain (16). We can only postulate that, since the *ndk::km* disruption only involves an insertion into the *ndk* gene and no deletion within the gene itself, some genetic event can occur randomly at low frequency that results in the restoration of the *ndk* gene. The protein sequencing analysis of the NDP kinase protein also provided a value for the absolute amount of the protein in a sample for which we had determined the level of ADP kinase activity, and allowed us to calculate a value for the turnover rate of approximately 500 sec.[-1] using our assay conditions. This value corresponds well with previously reported k_{cat} values for bacterial NDP kinases (20). It also allowed us to calculate a value of ~10 ng/µl for the amount of NDP kinase in the fraction of DnaK purified from *ndk*+ cells with the highest ADP kinase activity. This value was typically lowered to ~20 pg/µl when DnaK was purified from *ndk::km* cells and ~0.03 pg/µl following the nucleotide-removal treatment of DnaK purified from *ndk::km* cells.

In addition to a nonspecific NDP kinase activity, NDP kinase has an active ATPase activity (20). This activity can explain the high rates of ATP hydrolysis which exist in fractions with little DnaK. The kinetic profiles of MonoQ fractions 7 and 8 (Figure 4A) are particularly interesting in this regard. The large initial burst of P_i produced followed by a much slower rate of ATP hydrolysis initially puzzled us. The presence of NDP kinase explains these kinetics. NDP kinase rapidly hydrolyzes ATP, and when no NDP is available to be phosphorylated, releases free P_i. As ADP accumulates, NDP kinase has more substrate to phosphorylate, and the rate of P_i production slows. Finally, when the molar amounts of ATP and ADP are equal, NDP kinase shuffles gamma-phosphates from ATP to ADP with no net change in the nucleotide pool and no further accumulation of P_i. The rapid kinetics of ATP hydrolysis by NDP kinase makes accurate kinetic measurements of DnaK ATPase impossible in its presence. The presence of NDP kinase in DnaK preparations is likely a contributing factor for the wide range of k_{cat} values reported for DnaK ATPase and is probably the cause of observations of initial burst kinetics (1) and ADP-ATP exchange activity (10) in DnaK preparations. The NTP hydrolysis activity of NDP kinase is also nucleotide nonspecific (20), and its presence could account for the previous report of GTP, CTP, and UTP hydrolysis by DnaK with rates lower than the rate of ATP hydrolysis (15).

Neither the nature of the multiple states of DnaK revealed by MonoQ chromatography nor the reason that the nucleotide-removal treatment converts them into one form is known. It is possible that the extensive dialysis involved in the treatment itself is sufficient for this conversion, as we have not tested the ability of extensive dialysis alone to elicit this change. The multiple states are not the result of covalent modifications, truncations, different nucleotide bound states, or multimeric states of DnaK. The states may simply be DnaK molecules with different polypeptide substrates bound. One would predict that the exposure of DnaK to the high concentrations of ATP during its purification would result in the release of any bound peptides, although they could perhaps rebind at a later stage in the purification protocol.

DnaK has a weak physical interaction with his-tagged NDP kinase. The significance of this interaction is not known. DnaK, since it is a molecular chaperone, binds a wide range of

substrate peptides (23). The weak interaction with his-tagged NDP kinase could simply reflect the peptide binding chaperone function of DnaK. It is also not known what effect the histidine tag on the NDP kinase protein has on this interaction and whether DnaK would bind unmodified NDP kinase with higher, lower, or equal affinity. However, DnaK is one of only two proteins that can be detected by a Coomassie stained SDS-PAGE gel to co-elute with his-tagged NDP kinase. This physical interaction, regardless of its significance, may be the reason for the co-purification of small but significant amounts of NDP kinase with DnaK.

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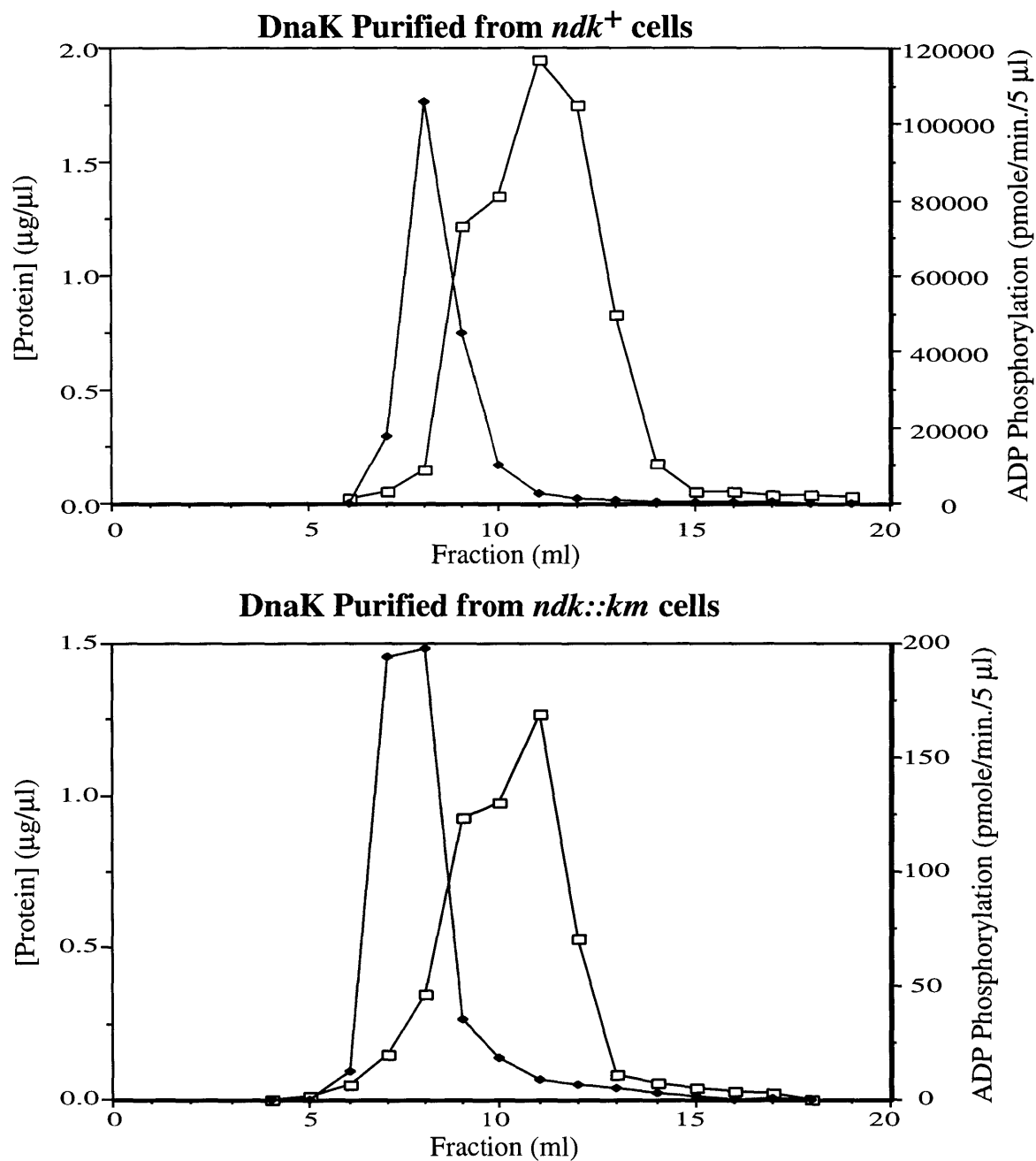
Figure 1. Chromatograms of Protein Concentration and ADP Kinase Activity from MonoQ Separation of DnaK

DnaK overexpressed in either *ndk*+ or *ndk::km* cells was purified according to the standard purification protocol. Fractions from the final chromatographic step (MonoQ anion-exchange column) were collected and assayed for total protein concentration (□) and ADP kinase activity (•) Note the difference in scale between the two panels.
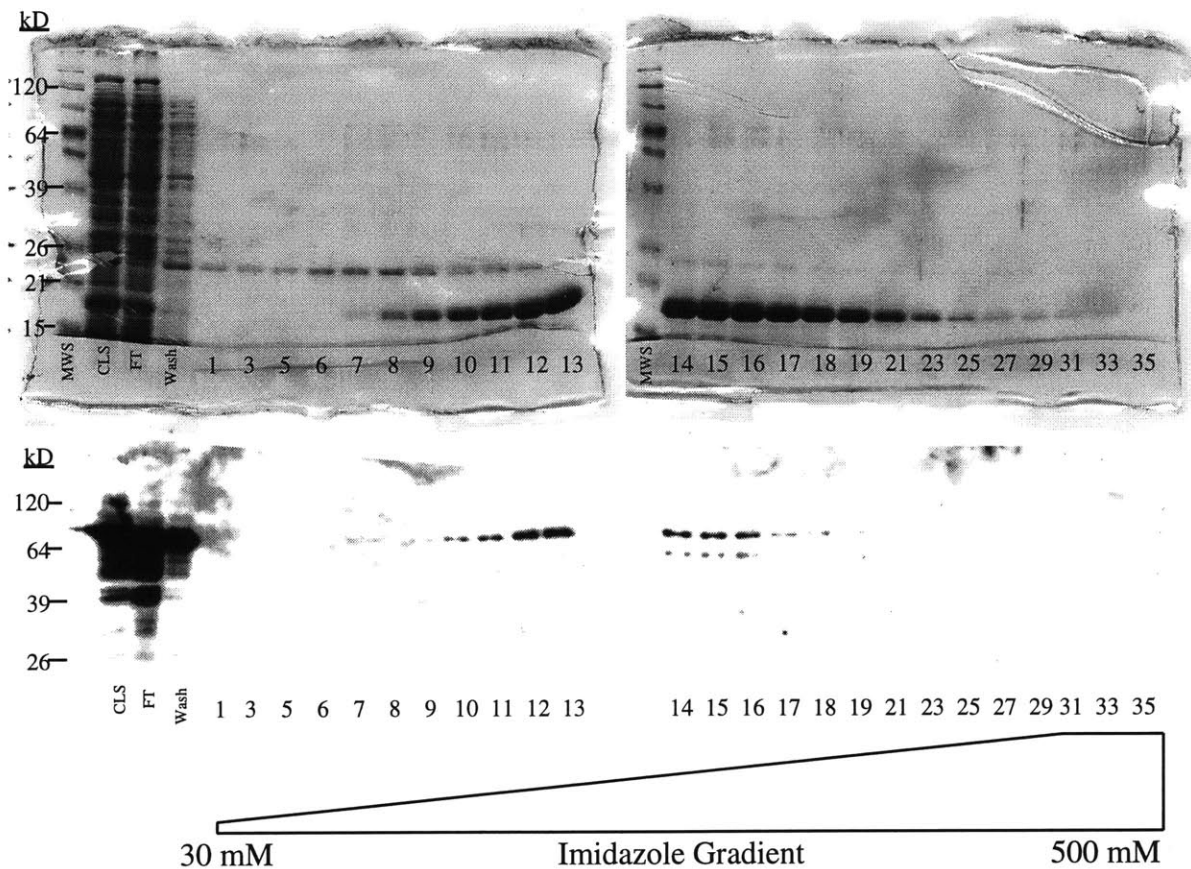
Figure 2. Copurification of DnaK with Ndk-N-his

TB2000 cells were grown, induced to overexpress Ndk-N-his, and lysed by sonication. The cell lysate supernatant was applied to a His-Bind IMAC column. The column was washed and eluted with a 30 mM - 500 mM imidazole gradient. Shown above are Coomassie stained SDS-PAGE gels (top) and Western Blots of SDS-PAGE gels identical to the Coomassie stained gels above probed with α-DnaK antibody (bottom). The lanes are labeled as follows: MWS , molecular weight standards ; CLS , crude lysate supernatant (5 µl) ; FT , IMAC flow through (5 µl) ; Wash , IMAC wash (5 µl) ; numbers ; eluent fraction number (Fraction 1 - 30 mM imidazole to Fraction 30 - 500 mM imidazole over a linear gradient, Fraction 30 to Fraction 35 - 500 mM imidazole) (5 µl each fraction).

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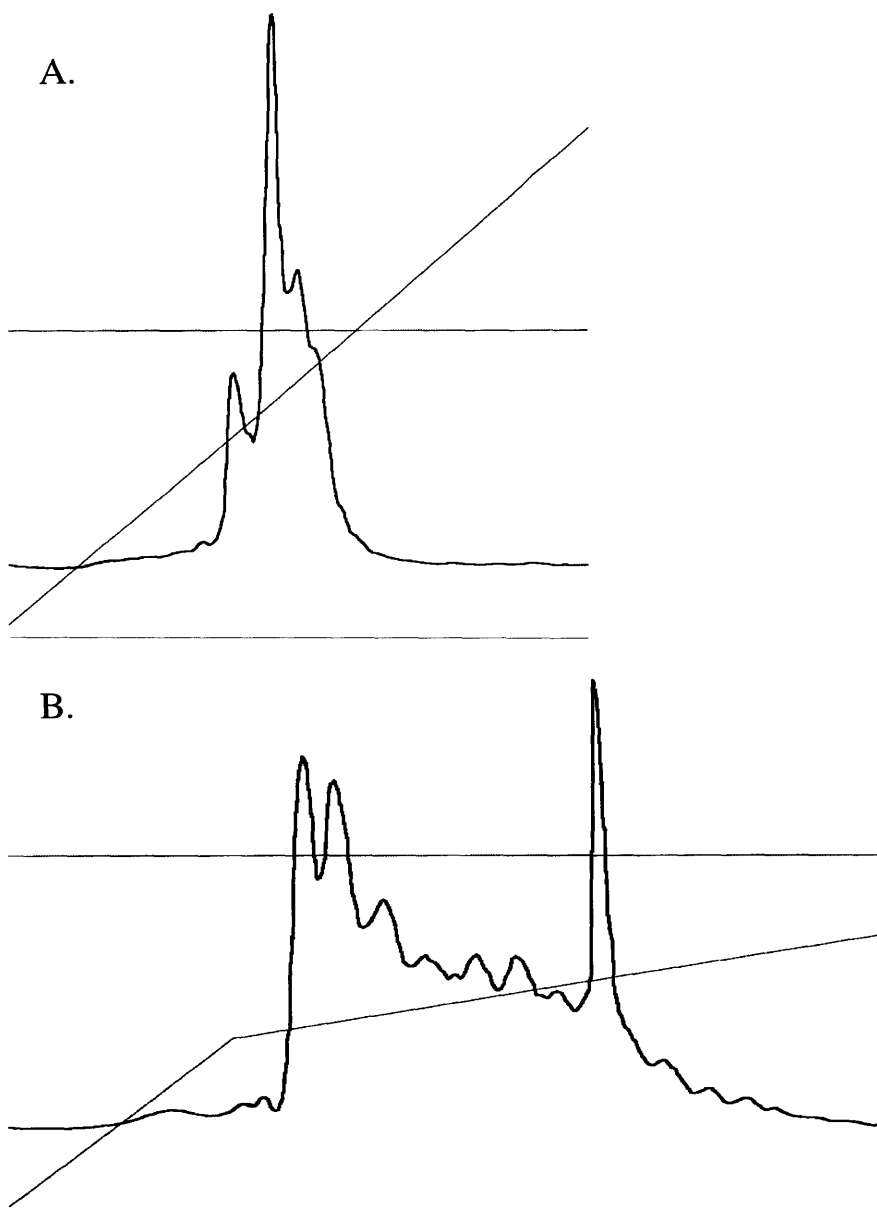
Figure 3. Chromatograms of the MonoQ Separation of DnaK

DnaK was purified according to the standard purification protocol. For the MonoQ separation, the gradient of KCl was run as follows:

      (A) Single linear gradient of 50 mM to 750 mM over 30 ml.
      (B) Double linear gradient of 50 mM to 225 mM over first 7.5 ml, 225 mM to 435 mM over next 45 ml.

The chromaograms above show traces of the absorbance at 280 nm and the KCl gradient. The horizontal lines represent 50 mM KCl (lower line) and 400 mM KCl (upper line).

A.

Fraction 6

Fraction 7

Fraction 8

Fraction 9

Fraction 10

Fraction 11

Fraction 12

Fraction 13

Fractions
14
15
16
17
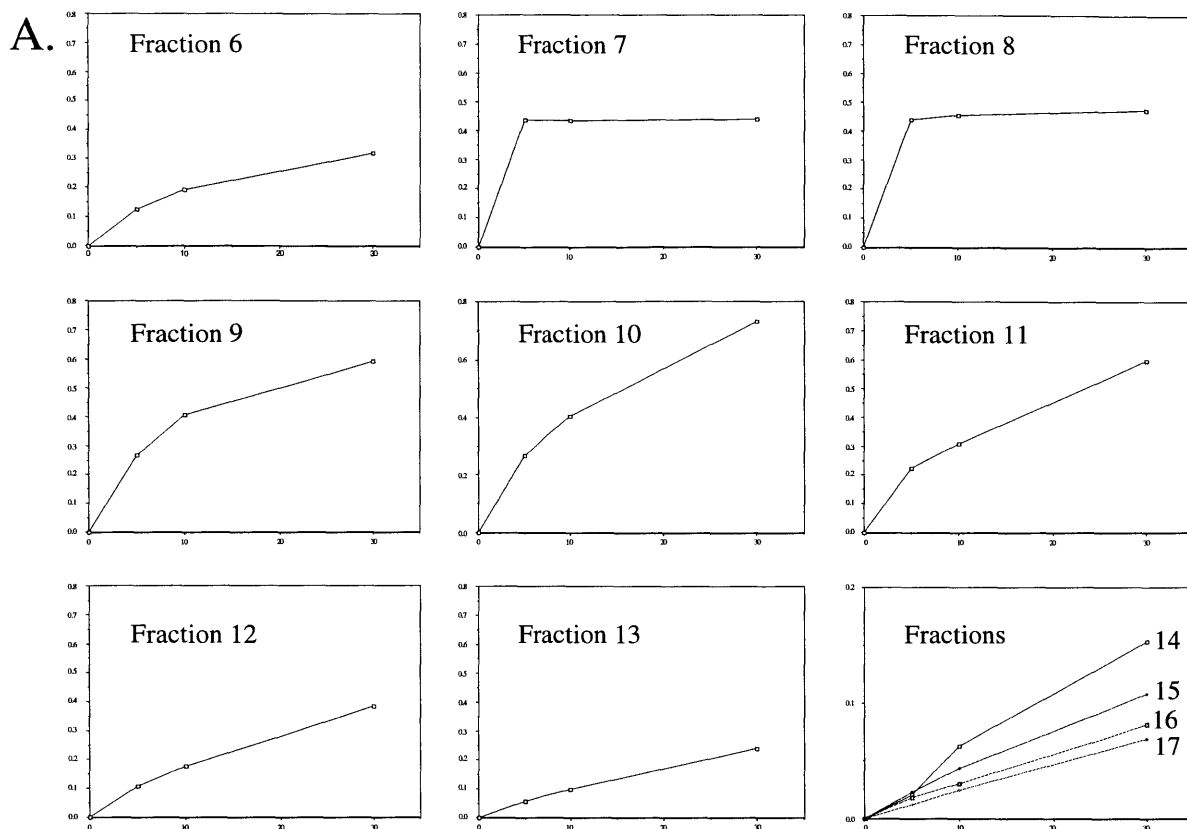
B.

MonoQ Fraction	[Protein] ($\mu g/\mu l$)	ATPase Rate (pmole/min./5 μl)		ADP Phosphorylation Rate (pmole/min./5 μl)
		Burst (0 - 5 min.)	Steady-State (5 - 30 min.)	
6	0.0500	25.0	7.62	12.3
7	0.150	86.7	0.119	194
8	0.350	87.3	1.35	198
9	0.924	53.0	12.9	35.0
10	0.976	53.0	18.4	18.7
11	1.27	44.2	14.8	8.98
12	0.532	21.2	11.0	6.30
13	0.0809	11.5	7.22	5.25
14	0.0528	4.13	5.25	3.08
15	0.0368	4.58	3.37	1.73
16	0.0251	3.61	2.50	0
17	0.0207	2.34	2.28	1.04

Figure 4. MonoQ Elution Profiles of ATPase Activity, ADP Kinase Activity and Protein Concentration for DnaK Purification

DnaK was purified from *ndk::km* cells following the standard purification protocol. MonoQ fractions were assayed for ATPase activity by adding 5 μl of the fraction to [γ-^{32}P]ATP (final concentration: 39.7 μM) in ATPase Buffer and incubating at 30°C for various times. The amount of P_i and ATP in each reaction was determined by TLC separation followed by phosphorimaging analysis. The graphs above (A) show the kinetic profile of P_i production by each MonoQ fraction. For each graph, the y-axis is $[P_i]/([ATP] + [P_i])$ and the x-axis is (continued on next page)
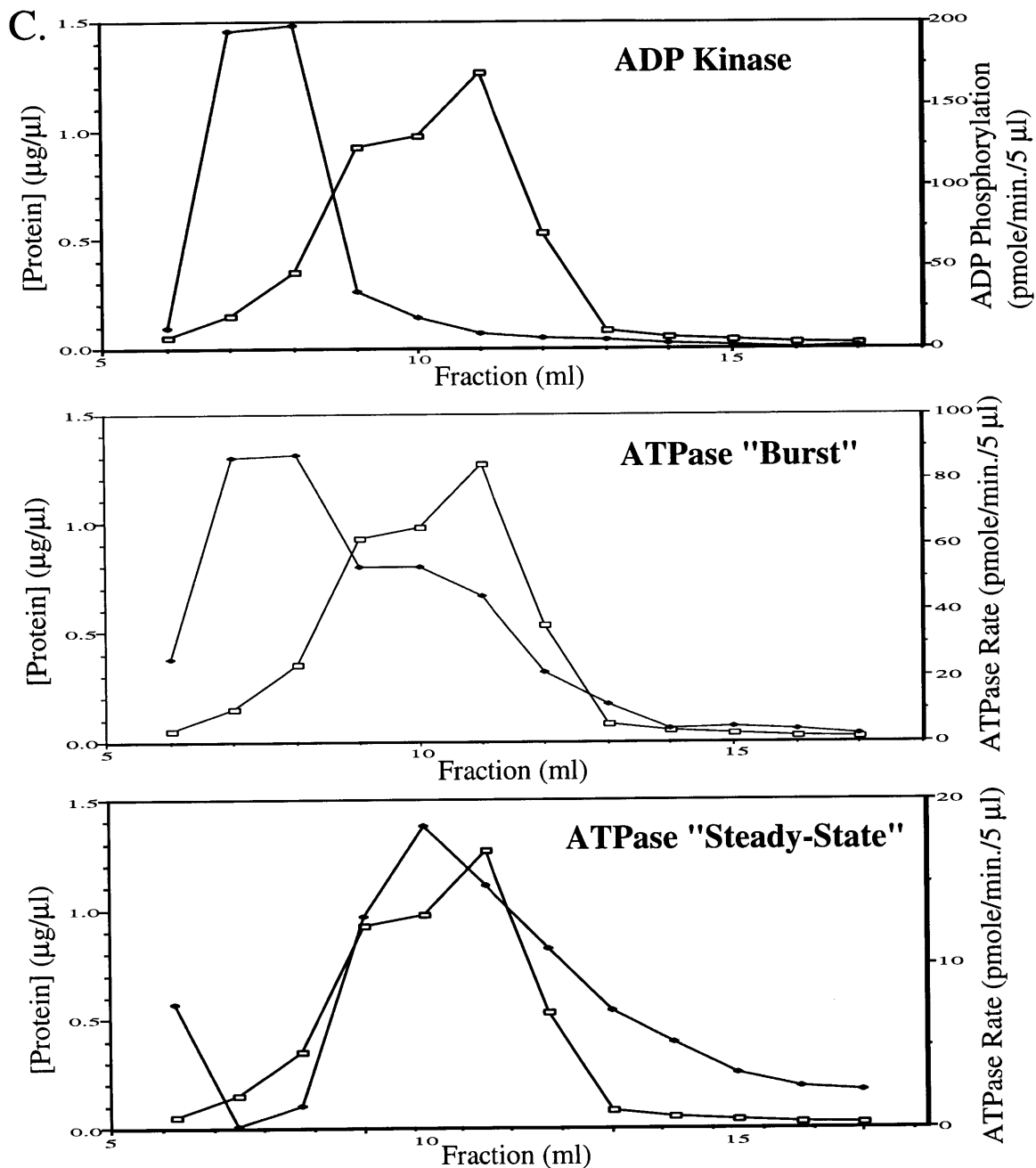
Figure 4 (continued).

time (minutes). The ATPase rate for each fraction was calculated in terms of the size of the initial burst by determining the rate over the first 5 minutes and in terms of the steady-state rate by determining the rate between minutes 5 and 30. This data is presented along with protein concentration and ADP kinase activity data (from Figure1) in the table (B). The data from this table was used to graphically portray the elution profile of each of these three activities (•) along with the elution profile of protein (□) from MonoQ (C).

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A.

B.

C.
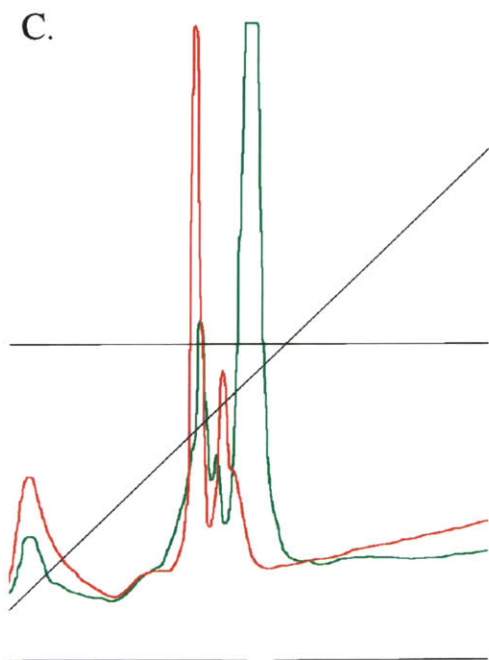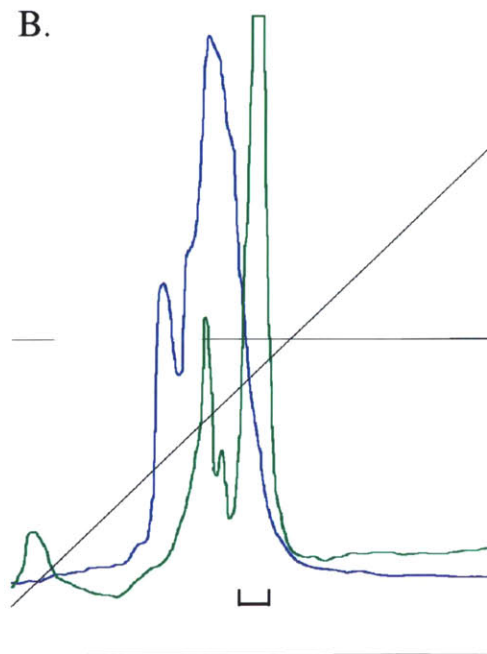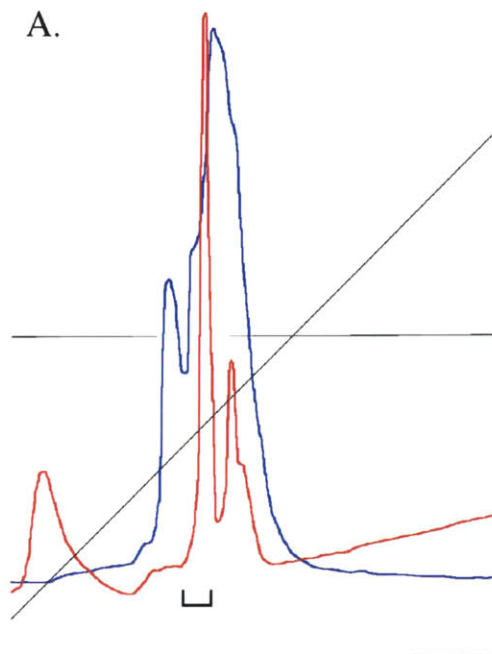
Figure 5. Chromatograms of Early and Late Eluting DnaK Reinjected onto MonoQ column

Early and late eluting MonoQ fractions of DnaK were collected and reinjected onto a MonoQ column. Shown are the chromatograms from the MonoQ separation of DnaK purified by the standard protocol (blue), early eluting DnaK from the blue chromatogram (black bracket in (A)) reinjected onto MonoQ (red), and late eluting DnaK from the blue chromatogram (black bracket in (B)) reinjected onto MonoQ (green). Figures A, B, and C show each possible pair of the chromatograms for comparison. A linear gradient of KCl from 50 mM to 750 mM over 30 ml was run for each separation. The colored lines are traces of the absorbance at 280 nm and the black lines are the KCl gradient. The horizontal lines represent 50 mM KCl (lower line) and 400 mM KCl (upper line).
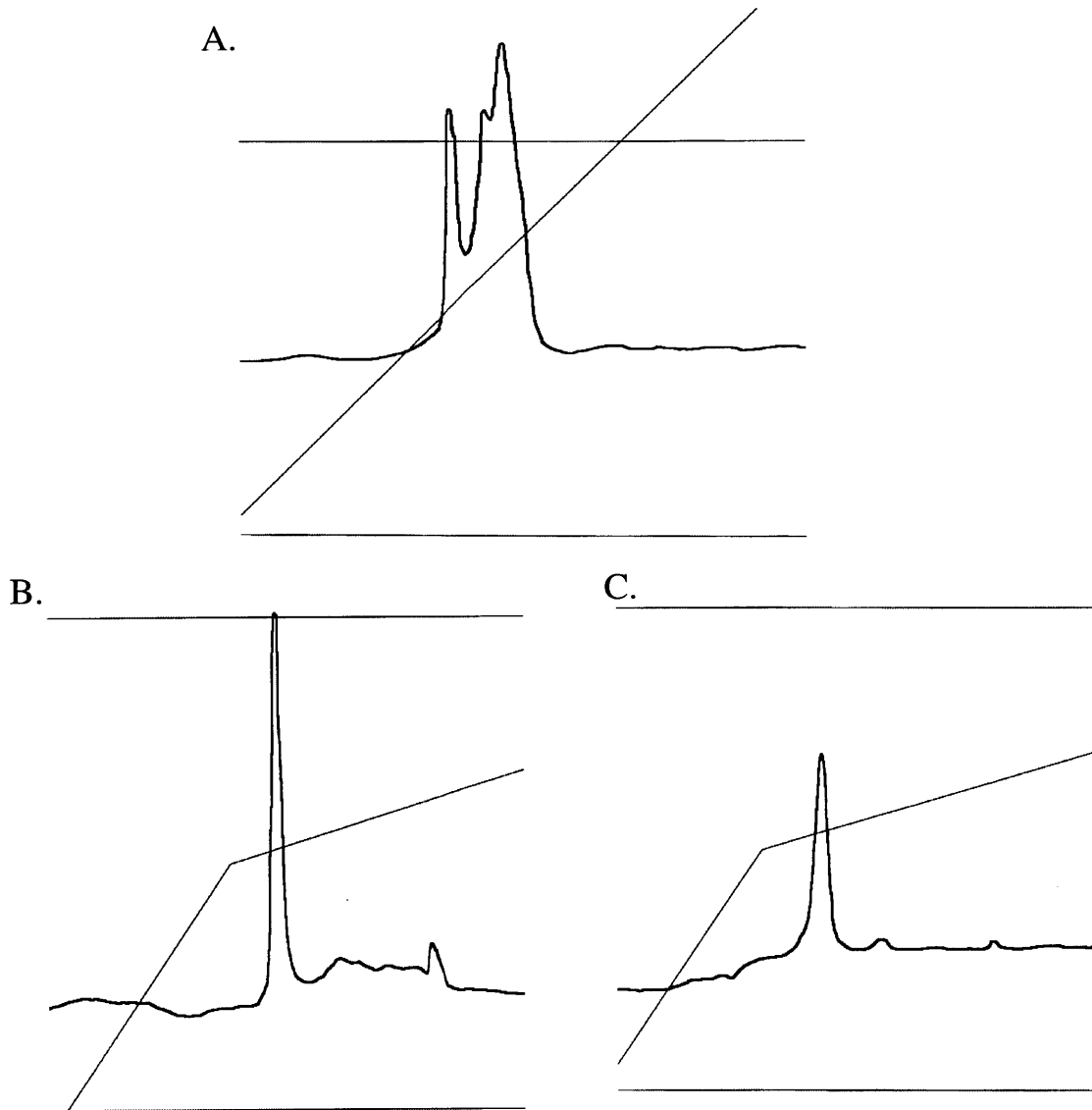
Figure 6. Chromatograms of DnaK Separated by MonoQ Following Removal of Nucleotide and Following Addition of ATP to Nucleotide-Free DnaK

The following MonoQ chromatograms are shown:
(A) DnaK purified according to the standard purification protocol.
(B) DnaK from the chromatogram in (A) pooled, saturated with AMP-PNP, and dialyzed extensively to remove nucleotide.
(C) Nucleotide-free DnaK from (B) following incubation in 2 mM ATP.
The gradient of KCl was run as follows:
(A) Single linear gradient of 50 mM to 750 mM over 30 ml.
(B) & (C) Double linear gradient of 50 mM to 225 mM over first 7.5 ml, 225 mM to 435 mM over next 45 ml.
The chromaograms above show traces of the absorbance at 280 nm and the KCl gradient. The horizontal lines represent 50 mM KCl (lower line) and 400 mM KCl (upper line).

A.

B.
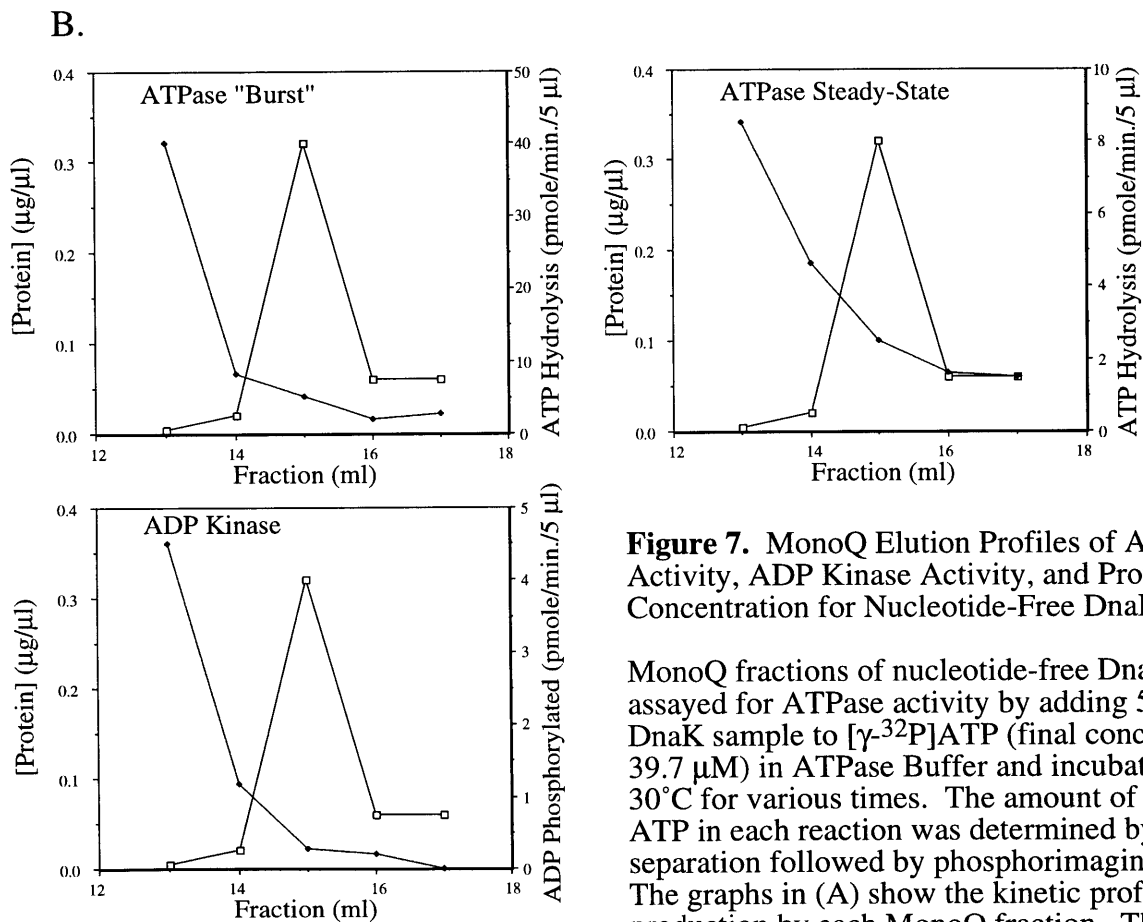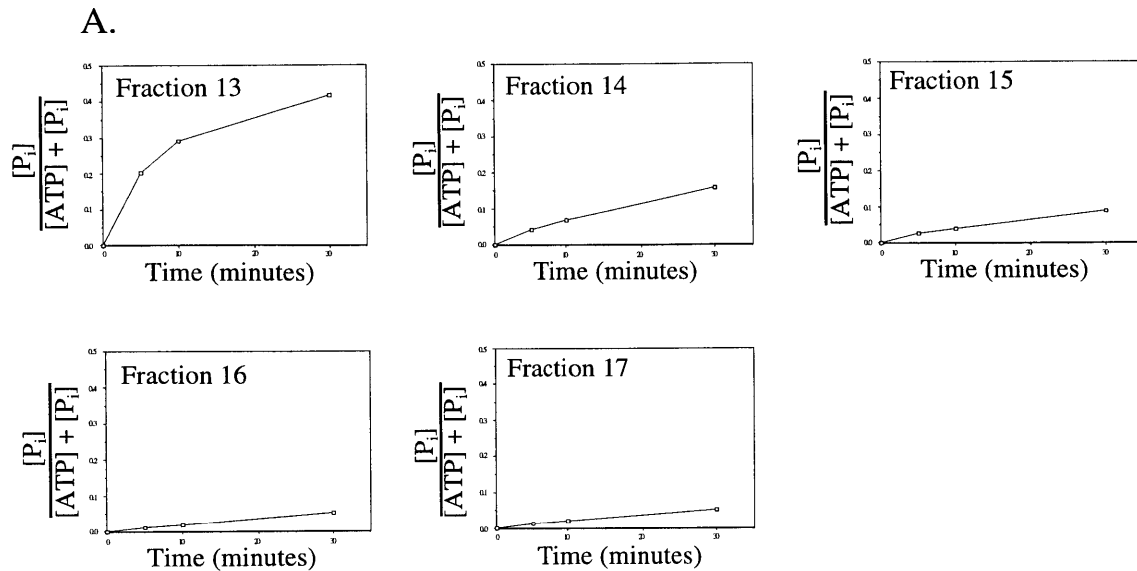
Figure 7. MonoQ Elution Profiles of ATPase Activity, ADP Kinase Activity, and Protein Concentration for Nucleotide-Free DnaK

MonoQ fractions of nucleotide-free DnaK were assayed for ATPase activity by adding 5 μl of DnaK sample to [γ-^{32}P]ATP (final concentration: 39.7 μM) in ATPase Buffer and incubating at 30°C for various times. The amount of P_i and ATP in each reaction was determined by TLC separation followed by phosphorimaging analysis. The graphs in (A) show the kinetic profile of P_i production by each MonoQ fraction. The ATPase rate for each fraction was calculated in terms of the size of the initial burst by determining the rate over the first 5 minutes and in terms of the steady-state rate by determining the rate between minutes 5 and 30. The elution profiles (B) of these two activities (.) as well as the ADP kinase activity (.) is shown along with the elution profile of protein (□) from MonoQ.

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Chapter 4

Biochemical Analysis of DnaK K70A and T199S Mutants:

Two New Classes of DnaK Mutants

Abstract

We measured various biochemical activities of *Escherichia coli* DnaK protein and three DnaK protein mutants (T199A, T199S, and K70A) that were purified from *ndk::km* cells. The DnaK preparations that we used in the assays had only a very low background nucleoside diphosphate kinase activity, allowing more accurate measurements of the biochemical activities than had previously been possible. The ability of the various DnaK mutants to complement defects of *ΔdnaK* cells was also tested. DnaK mutants T199A and K70A were completely unable to complement the loss of DnaK function, while DnaK mutant T199S was partially successful at complementing the aberrant phenotypes of *ΔdnaK* cells. DnaK T199A and K70A proteins have defective ATPase and autophosphorylation activities. The steady-state rate of ATP hydrolysis by each of these mutants is reduced to approximately 3% that of wild type DnaK. The ATPase activity of the DnaK T199S protein is largely intact, with a k_{cat} of 0.120 min.$^{-1}$, compared to the k_{cat} of 0.158 min.$^{-1}$ for wild type DnaK. The DnaK T199S protein also retains approximately 13% of the autophosphorylation activity of wild type DnaK, while the autophosphorylation activities of the T199S and K70A mutants is completely abolished. The peptide binding and release activities of each DnaK protein were measured by using a fluorescently labeled peptide substrate. All four DnaK proteins bind the peptide, and wild type, T199A, and T199S DnaK proteins release the peptide with similar kinetics upon the addition of ATP. The DnaK K70A protein, in contrast, does not release the peptide upon the addition of ATP. Measurements of the fluorescence of the single tryptophan residue of DnaK shows that ATP induces a conformational change in the wild type, T199A, and T199S DnaK proteins, but not the DnaK K70A protein. The DnaK T199S and K70A proteins each represent a new class of DnaK mutant. The T199S mutant has near normal levels of ATPase activity and undergoes an ATP induced conformational change that results in the release of peptide, but it is not able to fully complement loss of DnaK function in the cell. The K70A mutant is defective in both ATP hydrolysis and ATP induced conformational change and peptide release.

Introduction

Hsp70 proteins are a highly conserved family of molecular chaperones, proteins that facilitate the folding of other proteins. Hsp70s are found in all prokaryotic cells and in most compartments of all eukaryotic cells (9). *Escherichia coli* has one primary member of the Hsp70 family, the DnaK protein. Many Hsp70 proteins, heat shock proteins of 70-kDa, have increased levels of expression following heat shock or other forms of physical stress to the cell (28). Other Hsp70s, such as Hsc70, are expressed constitutively and are present at roughly the same level regardless of the physical state of the cell (33). Both heat-induced and constitutively expressed Hsp70 proteins play vital roles in the cell under both normal and heat shock conditions. The processes in which Hsp70 proteins function include the folding of nascent polypeptides (20), intracellular protein trafficking and membrane translocation (8), protein degradation (38), and disassembly of protein complexes (40). As molecular chaperones, Hsp70 proteins bind unstable or unfolded states of polypeptides, protect them from aggregation and denaturation, and help them along their folding pathway (3, 7, 14, 18, 19).

All Hsp70 proteins have a weak ATPase activity that is functionally linked to the cycles of peptide binding and release that characterize the chaperone activity (23). The ATP binding and hydrolysis activity is associated with the amino-terminal 44-kDa tryptic fragment of the protein (11). This region is extremely conserved at the level of amino acid sequence from *E. coli* DnaK to human Hsp70. The tertiary structures of this domain are highly conserved as well, and the crystallographic structure of the amino-terminal ATPase domain of *E. coli* DnaK (17) is nearly identical to that of bovine Hsc70 (11) and human Hsp70 (37). The biochemical mechanisms involved in ATP binding and hydrolysis are believed to be very similar, if not identical, for all Hsp70 proteins (12).

Hsp70 proteins also have a weak, calcium dependent autophosphorylation activity (42). The phosphorylated residue of DnaK is the threonine at position 199 (26), which is a completely conserved residue among Hsp70s and homologous to residue threonine-204 of Hsc70.

Substitution of threonine-199 of DnaK with alanine, valine, or aspartic acid results in a protein with no autophosphorylation activity and a greatly reduced ATPase activity (26). These DnaK mutants also fail to complement the loss of DnaK function in Δ*dnaK* cells (27). Structural studies with Hsc70 showed that this conserved threonine residue is not obligatory for ATP hydrolysis (30). Lysine-71 of bovine Hsc70, on the other hand, was found to be an essential residue for the chemical hydrolysis of ATP by the 44-kDa amino-terminal fragment (29). This residue is also completely conserved in the Hsp70 family and is homologous to lysine-70 of DnaK. This lysine residue is the only residue to date to be identified as essential for ATP hydrolysis.

In this study, we carried out biochemical and functional characterizations of DnaK and DnaK mutants with substitutions of residues threonine-199 and lysine-70. Based on our previous findings that nucleoside diphosphate kinase (NDP kinase) is present at very low levels in DnaK preparations and can result in inaccurate kinetic measurements, we made certain that our preparations were as free from NDP kinase as possible by purifying DnaK from *ndk::km* cells and using an extended protocol. We constructed and characterized a new DnaK derivative in which threonine-199 is replaced by serine to determine what effect a conservative substitution of this residue has on DnaK function. We also constructed and characterized a new DnaK derivative in which lysine-70 is replaced by alanine. We found that this K70A mutation results in a DnaK protein with a defective ATPase activity and which does not release peptide or undergo a conformational change upon the binding of ATP.

Materials and Methods

Reagents and Media. Reagents and media were as described in Chapter 2 and Chapter 3 of this thesis. In addition, N-((2-(iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (I-ANBD) was purchased from Molecular Probes (Eugene, OR), trichloroacetic acid (TCA) and trifluoroacetic acid (TFA) were purchased from J.T. Baker, acetonitrile was purchased from Fisher Scientific, and acetone was purchased from Sigma.

Strains and Plasmids. The *Escherichia coli* strains and plasmids used in this work are listed with their relevant features in Table 1. Plasmids were transformed by the standard $CaCl_2$/heat shock procedure (35). Strains TB3200 and TB3500 were constructed as described in Chapter 3 of this thesis.

Plasmid Constructions. Mutagenesis was performed using the Sculptor *in vitro* mutagenesis system (Amersham) following the standard protocol. All other DNA manipulations were performed as described (35). The plasmid pJM11 (27), which is a pBS derivative with the *dnaKdnaJ* operon under P_{lac} control and a BamHI site introduced 230 bp upstream from the start codon, was used as a parent plasmid for all constructions. Site-directed mutagenesis was used to change pJM11 (pBS-P_{lac}*dnaK*$^+$ *dnaJ*$^+$) to pTB104 (pBS-P_{lac}*dnaK*(T199S) *dnaJ*$^+$) and pTB107(pBS-P_{lac}*dnaK*(K70A) *dnaJ*$^+$). HindIII digestion followed by religation removed the *dnaJ* gene from these plasmids to create pTB124 (pBS-P_{lac}*dnaK*(T199S)) and pTB127 (pBS-P_{lac}*dnaK*(K70A)). The P_{lac}*dnaK*(T199S) *dnaJ*$^+$ BamHI fragment of plasmid pTB104 was subcloned into the pBR322 BamHI site to create plasmid pTB204 (pBR322-P_{lac}*dnaK*(T199S) *dnaJ*$^+$).

DnaK Purification. All DnaK samples used in this study were purified by an extended purification protocol. DnaK and its derivatives were purified from the *ndk::km* cells TB3200,

TB3221, TB3222, and TB3223 (see Table 1) using the same protocol described in Chapter 2 and Chapter 3 of this thesis. Following this standard purification procedure, the DnaK samples were treated to remove nucleotide by saturating the samples with AMP-PNP followed by MonoQ chromatography as described in Chapter 3 of this thesis.

Synthesis of PepH-ANBD. PepH (NRLLLCG) was synthesized by the M.I.T. Biopolymers Laboratory using a peptide synthesizer (Applied Biosystems, 430A). 5.0 mg PepH (6.3 µmole) in 0.5 ml 5.0 mM Tris buffer, pH 8.0 was combined with 2.5 mg I-ANBD (6.3 µmole) in 0.5 ml acetonitrile and incubated at 37°C for one hour. The modified PepH (PepH-ANBD) was purified by HPLC with a Waters Delta-pak C18 Cartridge column. The column was run at 2 ml/min. and a linear gradient of 0 - 80% acetonitrile in 0.1% TFA was run over 60 minutes. PepH-ANBD eluted from the column 6 minutes after unmodified PepH. The PepH-ANBD peak was collected and lyophilized.

ATPase Assays. Reaction mixtures (100 µl) contained Modified ATPase Buffer (50 mM Hepes-KOH, pH 7.6, 40 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 0.2 mg/ml ovalbumin), $[\alpha\text{-}^{32}P]ATP$, and DnaK. Radiolabeled ATP stocks were made by adding radiolabeled ATP to unlabeled ATP to give a final activity of 82.6 µCi/ml. The total concentration of ATP was recalculated following the addition of the radiolabeled ATP. The ATP and ATPase Buffer were mixed and preincubated at 30°C for 5 minutes, and hydrolysis was initiated by the addition of DnaK, marked as t=0 in the assays. For each DnaK sample, a range of ATP concentrations of 6.35 nM to 510 nM was used. DnaK was present in each reaction mixture at a concentration of 7.24 nM. The reaction was incubated in a water bath at 30°C and was stopped at 5, 10, and 20 minute time points by spotting 2 ml of the reaction onto a PEI-cellulose TLC plate (J.T. Baker Inc., Phillipsburg, NJ). Spotting the mixture on the TLC plate stopped the reaction immediately, as reactions quenched with 1 N HCl prior to spotting and reactions spotted without chemical quenching show the same extent of hydrolysis. The TLC plate was developed in 1 M Formic

Acid, 0.5 M LiCl, dried, and exposed to a Molecular Dynamics Storage Phosphor Screen. Data was obtained using a Molecular Dynamics PhosphorImager 445 Si and analyzed with ImageQuant 5.0. The amount each radiolabeled species present was determined by volume integration. The data was corrected for the level of background hydrolysis (typically 1% or less). The velocity of the reaction was determined by multiplying the value for ADP/(ATP + ADP) by the starting amount of ATP in the reaction and dividing by time.

Autophosphorylation Assays. Reaction mixtures (25 μl) contained buffer (40 mM Hepes-KOH, pH 7.6, 50 mM KCl, 10 mM CaCl$_2$), [γ-^{32}P]ATP (8.0 μM, 0.476 μCi/μl), and DnaK (0.929 μM). The reaction was incubated at 37°C for 30 minutes. Protein was precipitated with 20% TCA, 10 mM sodium pyrophosphate, and washed with 20% TCA followed by acetone. The sample were run on SDS-PAGE and the gel was exposed to a Molecular Dynamics Storage Phosphor Screen. Data was obtained using a Molecular Dynamics PhosphorImager 445 Si and analyzed with ImageQuant 5.0. The amount radiolabel in each DnaK band (if any) was determined by volume integration.

λ Sensitivity Assays. Strains GW8305, GW8309, TB1009, and TB1031 (see Table 1) were transduced with the *ΔdnaK52* allele as described (27). Overnight cultures of each of these strains were grown at 30°C in LB with ampicillin, kanamycin, and chloramphenicol and subcultured (1:20) into LB media with ampicillin, kanamycin, and chloramphenicol plus 1 mM IPTG, 0.2% maltose, and 8 mM MgSO$_4$. Following a two hour incubation at 30°C, 100 μl of each incubation was mixed with 3 ml λ top agar with 1 mM IPTG, 0.2% maltose, and 8 mM MgSO$_4$. The top agar was poured onto LB agar plates and allowed to solidify. Serial dilutions of λcI$^-$ phage were spotted (2 μl each) onto the plates. The plates were incubated at 30°C, overnight and inspected for clearing of cells on the bacterial lawn.

Fluorescence Measurements. Fluorescence measurements were performed using a FloroMax-2 spectrofluorimeter (ISA Jobin Yvon-Spex Instruments, S.A., Inc. (Edison, NJ)). For PepH-ANBD binding studies, the excitation wavelength was set at 480 nm. For DnaK conformational analysis, the excitation wavelength was set at 295 nm. For both sets of experiments the slit widths were set at: excitation - 5 nm, emission - 7 nm. All measurements were taken at 22°C.

Peptide-Release Kinetics. The kinetics of PepH-ANBD release from DnaK were measured on a Applied Photosystems stopped-flow spectrofluorimeter with a dead time of 1 ms. Reactions were initiated by mixing equal volumes (~70 µl). The excitation wavelength was set at 480 nm, and a 495 nm cutoff filter was used. The slit widths were set at: excitation - 0.5 mm, emission - 0.5 mm. All measurements were taken at 22°C.

DnaK Concentration Assays. The concentration of DnaK samples was determined as described in Chapter 2 of this thesis.

Results

ATPase and Autophosphorylation Activities of DnaK Mutants

DnaK has a very weak intrinsic ATPase activity (42) and is autophosphorylated on a conserved threonine residue (26). Nonconservative substitution of the threonine residue which becomes autophosphorylated (threonine-199) to alanine, valine, or aspartic acid results in a protein with greatly reduced ATPase activity and no autophosphorylation activity (26). These DnaK mutants also fail to complement a variety of abnormal phenotypes associated with $\Delta dnaK$ cells (27). In order to further investigate the role of threonine-199 in the biochemical and physiological functioning of DnaK, we studied the properties of DnaK in which a more conservative change of the threonine-199 residue to serine was made. In addition, our studies of the ADP kinase activity in DnaK preparations drew our attention to a report regarding mutation of lysine-71 in Hsc70. Lysine 71 of Hsc70 is a residue that is essential for the hydrolysis of ATP by the 44-kDa amino-terminal ATPase domain of Hsc70 (29) and is the first residue of any Hsp70 protein or Hsp70 ATPase domain which has been found to be obligatory for ATP hydrolysis. This lysine is conserved in all Hsp70 proteins and is homologous to residue lysine-70 of DnaK. Initially, we were interested in studying DnaK mutated at lysine-70 to determine if the protein retained ADP kinase activity. Following the realization that the ADP kinase activity in DnaK and Hsp70 preparations is due to trace amount of NDP kinase, we used mutant DnaK with lysine-70 replaced with alanine in parallel with other DnaK mutants in biochemical assays.

The DnaK proteins used in the following biochemical assays were all purified by an extended protocol (see Materials and Methods) which includes steps to remove bound nucleotide from DnaK followed by a second round of MonoQ chromatography. This purification protocol was used to ensure that all DnaK proteins were as free of NDP kinase activity as possible. All DnaK preparations used in the biochemical assays in this chapter had an ADP kinase activity of less than 0.025 pmole ADP phosphorylated per minute per pmole DnaK.

ATPase assays were performed with wild type DnaK as well as mutants with threonine-199 replaced with alanine (T199A) or serine (T199S) or lysine-70 replaced with alanine (K70A). A low concentration of DnaK (7.24 nM) was used in the assays to avoid the phenomenon of mutual depletion kinetics (16), which would result in the determination of erroneously high K_m values. For each reaction, the kinetics of ADP formation were followed and shown to be linear and without initial burst activity. The DnaK was incubated with ATP in concentrations ranging from 6.35 nM to 510 nM, the velocity of ATP hydrolysis was determined for each reaction, and Eadie-Hofstee plots were made of the data (Figure 1A). Figure 1B shows the values for V_{max}, k_{cat}, and K_m for the ATPase activities of DnaK, and the T199S, T199A, and K70A mutants determined from the Eadie-Hofstee plots. The amount of calcium-dependent autophosphorylation was also determined for each protein and expressed as a fraction of the amount of autophosphorylation that occurs in wild type DnaK. The turnover rate determined for wild type DnaK (0.158 min.$^{-1}$) is similar to some recently reported values (10, 36) and a few fold higher than some other recently reported values (5, 21, 22, 25). The K_m value for wild type DnaK (27.5 nM) correlates well with other reports in which mutual depletion kinetics were avoided (5). The K_m values of the three mutants are all within two-fold of the value for wild type DnaK. However, the turnover rate for the T199A mutant is only 3% that of wild type and is similar to the previously reported value (26). Mutation of lysine-70, reported to be absolutely required for ATP hydrolysis by the amino-terminal ATPase domain of Hsc70 (29), results in a DnaK protein with a significantly lowered, but not abolished ATPase activity. The k_{cat} of DnaK K70A is 3.5% that of wild type DnaK. Also significant is the finding that most of the wild type ATPase activity is preserved when residue threonine-199, which is completely conserved in the Hsp70 family, is replaced by serine. This mutant is also the only one with a residual autophosphorylation activity, measured to be 12.9% that of wild type DnaK. The K70A mutant, in which the site of autophosphorylation (threonine-199) is preserved, has no autophosphorylation activity.

DnaK Mutants Do Not Complement Loss of DnaK Function

The ability of each mutant to complement the loss of DnaK function was measured. Cells containing a deletion of the *dnaK* gene display a number of aberrant phenotypes including insensitivity to λ phage, inability to grow at temperatures of 42°C or higher, and extensive filamentation of the cells. GW8301 cells were transformed with pBR322 plasmids carrying the *dnaK* or mutant *dnaK* gene under P$_{lac}$ control. The Δ*dnaK52* allele was transduced into each of these strains, and the strains were grown in the presence of 0.5 mM IPTG to induce the expression of DnaK or mutant DnaK. DnaK T199A as previously described (27) and DnaK K70A fail to provide DnaK function, while DnaK T199S only partially complements the loss of DnaK. Figure 2A shows the cell morphology of Δ*dnaK* cells carrying only pBR322, pJM41 (pBR322-*dnaK*$^+$), pTB204 (pBR322-*dnaK*(T199S)), and pJM43 (pBR322-*dnaK*(T199A)). Expression of DnaK$^+$ complements the filamentation defect, while DnaK T199S only partially complements and DnaK T199A fails to complement the defect. DnaK T199S is also partially successful at complementing the λ insensitivity and temperature sensitivity defects of Δ*dnaK* cells (Figure 2B). Judged by the number of pfu of λcI$^-$ phage required to cause detectable clearing of cells on a bacterial lawn, DnaK T199S expressing cells are 100 times less sensitive to λ phage than DnaK$^+$ expressing cells. Cells expressing DnaK T199S also show weak growth at 42°C. The DnaK T199A and DnaK K70A expressing Δ*dnaK* cells, by contrast, show no λ sensitivity or growth at 42°C. Thus, the DnaK T199S protein, but not the DnaK T199A or DnaK K70A proteins, is at least partially able to perform the role of DnaK in the cell.

ATP Does Not Induce DnaK K70A To Release Peptide

Short peptides can be used to study the interaction of Hsp70 proteins with polypeptide substrate (13). We used a derivative of the seven amino acid Peptide NR (NRLLLSG), which has been shown to bind to DnaK with high affinity (15), called PepH (NRLLLCG) as a peptide substrate for DnaK. A cysteine-specific fluorescent probe, I-ANBD, was covalently attached to PepH, allowing it to be used for kinetic binding studies. The intensity of the fluorescent signal of

PepH-ANBD increases when it binds to DnaK, allowing for a direct assay of the extent of binding (41). DnaK and the T199S, T199A, and K70A mutants were incubated with PepH-ANBD and binding was measured using a fluorescence spectrometer. Figure 3 shows that when PepH-ANBD is mixed with DnaK or any of the three mutants, the emission spectra signal (blue) has a greater intensity than the emission spectra of an equal amount of PepH-ANBD alone (black), showing that PepH-ANBD binds to all of the proteins. The addition of ATP results in the rapid release of peptide by DnaK (36). ATP was added to the various DnaK proteins with bound PepH-ANBD. The addition of ATP results in the reduction of fluorescence signal by the PepH-ANBD (red) almost to the level of PepH-ANBD alone for wild type DnaK, DnaK T199S, and DnaK T199A, but results in very little change in the fluorescence signal of PepH-ANBD bound to DnaK K70A. These results indicate that the addition of ATP results in the nearly complete dissociation of PepH-ANBD from DnaK, DnaK T199S, and DnaK T199A, but does not result in the dissociation of PepH-ANBD from DnaK K70A.

The kinetics of the release of PepH-ANBD by DnaK upon the addition of ATP were also measured. The reaction was initiated in a stopped-flow device by rapidly mixing ATP with prebound complexes of DnaK and PepH-ANBD. Figure 4 shows traces of the fluorescence signal over time for DnaK$^+$, T199S, T199A, and K70A with PepH-ANBD bound following rapid mixing with ATP. The decreasing fluorescence signal shows that DnaK$^+$, DnaK T199S, and DnaK T199A all release PepH-ANBD upon the addition of ATP with relatively rapid and similar kinetics. The fluorescence signal for PepH-ANBD bound to DnaK K70A, by contrast, remains flat, indicating that ATP does not induce DnaK K70A to release peptide.

ATP Does Not Induce DnaK K70A To Undergo a Conformational Change

The binding of ATP induces a global conformational change in DnaK which results in the release of bound peptide (23). DnaK contains a single tryptophan residue (tryptophan-102), the fluorescence of which changes upon the addition of ATP to DnaK (1). This fluorescence shift is the result of the global conformational change that occurs when DnaK binds ATP and can be used

to observe the conformational changes of DnaK. The fluorescence spectra of DnaK, DnaK T199S, DnaK T199A, and DnaK K70A were collected in the presence and absence of ATP by exciting the samples with light at a wavelength of 295 nm in a fluorescence spectrometer. Figure 5 shows that for DnaK, DnaK T199S, and DnaK T199A, the fluorescence spectrum in the presence of 50 μM ATP (blue) is reduced in intensity and slightly shifted to shorter wavelengths compared to the fluorescence spectrum without ATP added (black). These results indicate that ATP induces a conformational change in these three proteins. The fluorescence spectra of DnaK K70A, however, are the same in both the presence and absence of ATP (Figure 5). There was no change in the spectrum even following prolonged incubation of DnaK K70A with ATP (45 minutes at room temperature). These results indicate that ATP does not induce a conformational change in DnaK K70A, at least not a change similar to the one that occurs in wild type DnaK and results in a change in the local environment of tryptophan-102 and its fluorescence spectrum. Therefore, ATP induces DnaK K70A to neither undergo a conformational change nor release peptide. Thus, this mutation is a new class that results in a protein that is defective in both ATP hydrolysis and ATP induced conformational change.

Discussion

We have biochemically characterized *E. coli* DnaK and derivatives with alterations at the threonine-199 and lysine-70 positions. The DnaK proteins used in this study were purified from *ndk::km* cells in order to prevent inaccuracies in the biochemical measurements due to the presence of NDP kinase in the preparations. The preparations were also treated to remove bound nucleotide using a procedure which we found further reduces the amount of co-purifying nucleoside diphosphate kinase activity (see Chapter 3 of this thesis). All four DnaK preparations used in this study had measurable, but very low, amounts of ADP kinase activity, ranging from 0.015 to 0.025 pmole ADP phosphorylated/min/pmole DnaK. This level of ADP kinase activity would be caused by approximately 5×10^{-7} pmole of NDP kinase per pmole of DnaK. This is the first study of which we are aware in which an effort was made to reduce and monitor the amount of co-purifying NDP kinase activity in a DnaK or Hsp70 preparation in order to ensure the validity of the biochemical assays.

We found that wild type DnaK and DnaK T199S have ATPase activities with rates about 30 times higher than those of DnaK T199A and DnaK K70A. The k_{cat} values for the ATPase reaction were found to be 0.158 min.$^{-1}$, 0.120 min.$^{-1}$, 0.00481 min.$^{-1}$, and 0.00558 min.$^{-1}$ for DnaK$^+$, T199S, T199A, and K70A respectively. Because the background amount of ADP kinase activity was nearly the same in all four DnaK preparations, these k_{cat} values represent real differences in the ATPase activities of DnaK$^+$ and DnaK T199S from those of DnaK T199A and DnaK K70A. We recognize that the low ATPase rates of the DnaK T199A and DnaK K70A proteins might actually represent the amount of background hydrolysis due to the small amount of NDP kinase in the preparations. Therefore, the ATPase activity of the DnaK T199A and K70A proteins may actually have k_{cat} values lower than those we assigned, or even be 0. However, since the amount of residual NDP kinase activity varied by less than two fold between the DnaK$^+$, T199S, T199A, and K70A preparations, the amount of NDP kinase dependent ATPase activity in the DnaK$^+$ and T199S preparations would not be expected to be significantly greater than that in

the T199A and K70A preparations. For this reason, the true k_{cat} values for the ATPase activities of DnaK$^+$ and DnaK T199S are at most ~0.006 min.$^{-1}$ lower than those we report in Figure 1.

The k_{cat} value that we determined for the ATPase activity of wild type DnaK (0.158 min.$^{-1}$) correlates well with previously reported values of 0.180 min.$^{-1}$ (31) and 0.130 min.$^{-1}$ (10, 36) but is several fold higher than other reports (5, 25, 34) and nearly ten fold higher than one report (32). The reason for these differences is not known. It is possible that despite our effort to obtain pure samples of DnaK, our preparations contain peptide fragments that stimulate the ATPase activity of DnaK. It is also possible that some fraction of the DnaK in preparations from other labs is inactive. The DnaK T199A mutant has previously been studied, and the k_{cat} value determined by us for the ATPase activity of this protein (0.00481 min.$^{-1}$) agrees with previously published values of 0.007 min.$^{-1}$ (26) and 0.011 min.$^{-1}$ (31). The K71A mutation in the 44-kDa amino-terminal ATPase fragment of Hsc70 was found to have no measurable ATPase activity (29). The small, but measurable, ATPase activity in our DnaK K70A preparation could be due to a difference between full length protein and the 44-kDa amino-terminal fragment, a difference between DnaK and bovine Hsc70, or the total absence of NDP kinase in the Hsc70 preparation. The complete lack of autophosphorylation by the DnaK T199A mutant that we observed has been previously reported (26). The lack of autophosphorylation by the DnaK K70A mutant is not surprising considering that the autophosphorylation activity is considered to be a side-reaction of ATP hydrolysis (12), and the mutant has very little ATPase activity.

It is interesting that the DnaK T199S mutant retains most of the ATPase activity of wild type DnaK, but is not able to fully function as DnaK in the cell as shown by the low sensitivity to λ phage, slow growth at 42°C, and partial filamentation of Δ*dnaK* cells expressing DnaK T199S. The T199S protein also undergoes a conformational change and releases peptide when mixed with ATP, so it does not appear to be defective in the coupling of the ATPase and peptide release activities. Threonine-199 of DnaK is completely conserved among Hsp70 proteins. While it was initially thought that phosphorylation of this threonine might play an important role in the reaction pathway of ATP hydrolysis, this notion was disproven by structural studies of mutants altered at

the threonine-204 (homologous to threonine-199 of DnaK) position of Hsc70 (30). The reason that the DnaK T199S protein is unable to fully perform DnaK function in the cell is not known, but it represents a new class of DnaK mutation: one with a functional ATPase activity coupled to a functional peptide release activity, but unable to complement the ΔdnaK mutation. Previously defined classes of DnaK mutations include those with defective ATPase activities, but functional peptide release activities such as DnaK T199A (31) and those with functional ATPase activities, but defective peptide release activities such as DnaK E171A (2).

The DnaK K70A derivative also belongs to a previously undefined class of mutant DnaK proteins, those with defective ATPase activities and defective peptide release activities. Structural studies of lysine-71 mutations in the 44-kDa amino-terminal ATPase fragment of bovine Hsc70 indicated that lysine-71 participates in the catalysis of ATP hydrolysis by stabilizing an H_2O molecule or OH^- ion for a nucleophilic attack on the gamma-phosphate of the bound ATP (29). The results of the Hsc70 study identified lysine-71 as the only residue yet known to be essential for the hydrolysis of ATP. Our results indicate that mutation of the homologous lysine in DnaK, lysine-70, results in a protein that does not undergo a conformational change or release peptide when mixed with ATP. Two possible explanations for this behavior are: 1) the K70A mutant does not bind ATP with the correct geometry required to elicit a conformational change, and 2) the K70A mutant, because it is unable to hydrolyze ATP, does not undergo a conformational change in the presence of ATP. No significant differences exist between the binding geometry of ATP to Hsc70 K71A (29) and ATP bound to other Hsc70 mutants that do change conformation upon the addition of ATP (12). Thus, the explanation that DnaK K70A does not undergo an ATP induced conformational change because it does not hydrolyze the bound ATP seems a viable possibility. We have previously described a model in which a rapid equilibrium between putative DnaK·ATP and DnaK*·ADP·P$_i$ forms exists. This equilibrium results in the ATP induced conformational change and peptide release by DnaK and is an attractive alternative to the model in which ATP binding alone results in a conformational change and peptide release. The appeal of our model is that it explains why nonhydrolyzable analogs of ATP do not elicit peptide release (23) and provides

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a structural explanation for the observed second step of ATP binding by DnaK (39). The observation that a mutation of a residue in DnaK that has been implicated in ATP hydrolysis in a homologous protein uncouples ATP binding from conformational change and peptide release lends support to this model.

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Table 1. List of Strains and Plasmids

Strain	Relevent Genotype	Source/Reference
MC4100	F⁻ *araD139 Δ(argF-lac)U169 rpsL150*(6) *relA1 deoC1 ptsF25 rpsR flbB301*	
BB1553	MC4100, *ΔdnaK52 sidB1*	(4)
GW8301	MC4100, pJM100	(27)
GW8302	BB1553, pJM100	(27)
GW8303	GW8301, pBR322	(27)
GW8305	GW8301, pJM41	(27)
GW8306	GW8302, pJM41	(27)
GW8309	GW8301, pJM43	(27)
TB1009	GW8301, pTB204	This work
TB1031	GW8301, pTB107	This work
JC7623	*recBrecCsbcB*	(24)
NA7623	JC7623, *ndk::km*	(24)
TB3200	NA7623, *ΔdnaK52*	This work
TB3500	JC7623, *ΔdnaK52*	This work
TB3220	TB3200, pJM6	This work
TB3221	TB3200, pTB127	This work
TB3222	TB3200, pJM8	This work
TB3223	TB3200, pTB124	This work

| Plasmid	Comments	Source/Reference
pBS		Stratagene
pBR322		New England Biolabs
pJM100	pACYC177, Km^r, lacI^q	(27)
pJM41	pBR322-P$_{lac}$*dnaK*$^+$ *dnaJ*$^+$	(27)
pJM43	pBR322-P$_{lac}$*dnaK*(T199A) *dnaJ*$^+$	(27)
pTB204	pBR322-P$_{lac}$*dnaK*(T199S) *dnaJ*$^+$	This work
pJM6	pBS-P$_{lac}$*dnaK*$^+$	(26)
pJM8	pBS-P$_{lac}$*dnaK*(T199A)	(26)
pTB124	pBS-P$_{lac}$*dnaK*(T199S)	This work
pTB127	pBS-P$_{lac}$*dnaK*(K70A)	This work

Figure 1. ATPase and Autophosphorylation Activities of DnaK and DnaK Mutants

(A) Eadie-Hofstee plots of ATPase activity for DnaK and DnaK mutants purified by the extended protocol. DnaK (final concentration: 7.24 nM) was incubated with $[\alpha\text{-}^{32}P]ATP$ (final concentration: 6.35 nM - 510 nM) in Modified ATPase Buffer at 30°C. The amounts of ADP and total nucleotide were determined following various incubation times as described in Materials and Methods, and the velocity of the ATPase reaction for each [ATP] was calculated. The line drawn on each graph in a best fit line determined from a least-squares linear-regression analysis of the data points.

(B) Table of ATPase activity parameters and the extent of autophosphorylation for DnaK and DnaK mutants. The V_{max}, k_{cat}, and K_m values were derived from the y-intercept and slope of the Eadie-Hofstee plots (A). The extent of autophosphorylation was determined as described in Materials and Methods. The extent of autophosphorylation of wild-type DnaK was set as 1.00.

A.
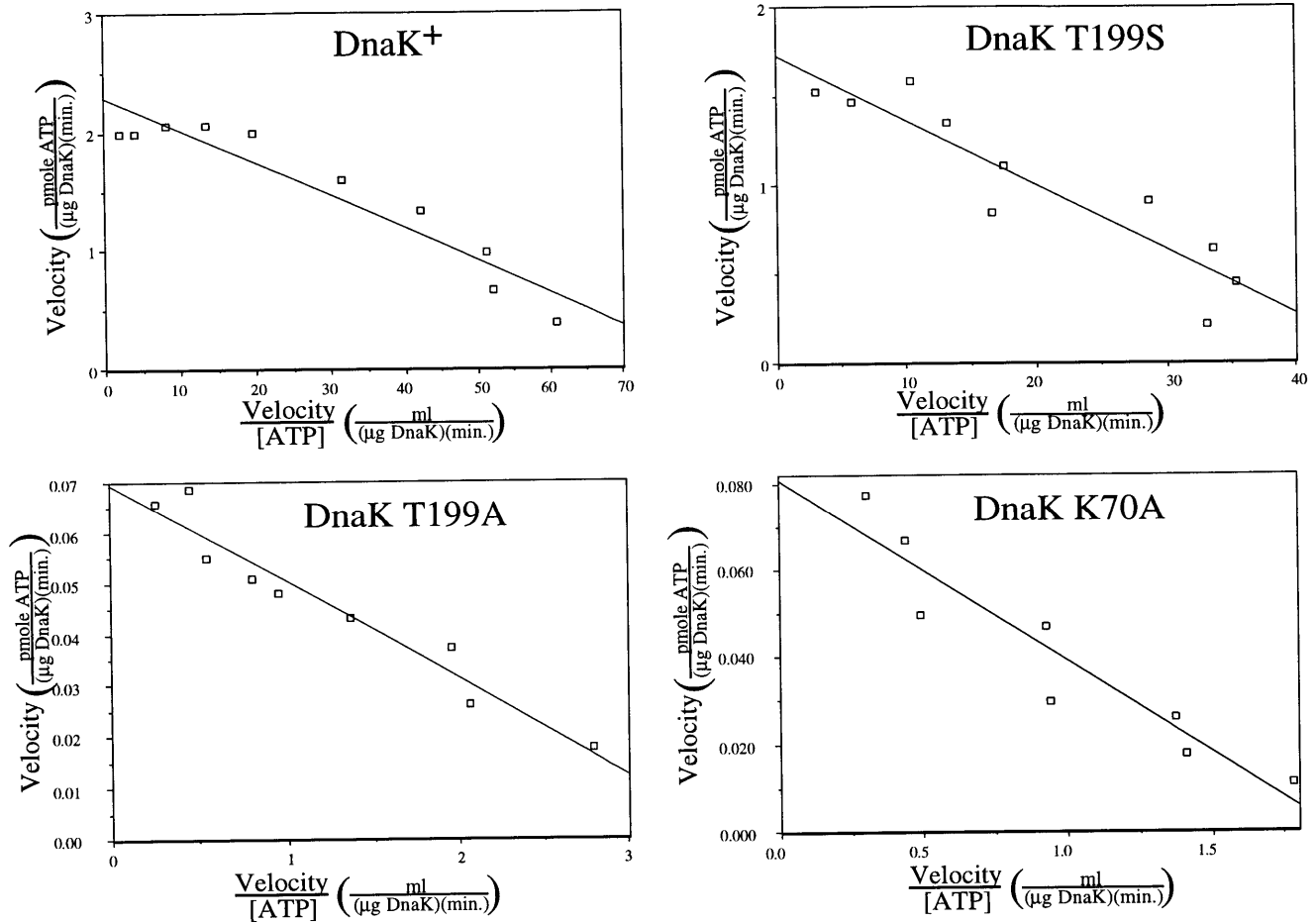
B.

DnaK	ATPase Activity			Extent of Autophosphorylation
	$V_{max}\left(\frac{\text{pmole ATP}}{(\mu g \text{ DnaK})(\text{min.})}\right)$	k_{cat} (min.$^{-1}$)	K_m (nM)	
wild type	2.29	0.158	27.5	1.00
T199S	1.73	0.120	36.5	0.129
T199A	0.0696	0.00481	19.1	0
K70A	0.0808	0.00558	41.8	0

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Figure 2. Phenotypes of *Escherichia coli* cells expressing DnaK mutants in a *ΔdnaK* background

(A) Cellular morphologies of cells expressing DnaK mutants. Strains expressing wild type or mutant DnaK in a *ΔdnaK* background were grown at 30°C on LB plates containing 0.5 mM IPTG, ampicillin, chloramphenicol, and kanamycin. Multiple colonies were picked for each strain and representative cells were photographed. Microscopic analyses were done on a Zeiss Axioplan Universal with Nomarski optics. The *ΔdnaK52* allele was transduced into the following strains: GW8303 (GW8301, pBR322), GW8305 (GW8301, pJM41 (*dnaK*$^+$)), GW8309 (GW8301, pJM43 (*dnaK*(T199A))), and TB1009 (GW8301, pTB204 (*dnaK*(T199S))) (see Table 1).

(B) λ sensitivity and temperature sensitivity of cells expressing DnaK mutants. The *ΔdnaK52* allele was transduced into strains GW8305, GW8309, and TB1009 as for (A), as well as TB1031 (GW8301, pTB107 (*dnaK*(K70A))) (see Table 1). λ sensitivity assays were performed as described and λ sensitivity is expressed as the number of pfu of λcI$^-$ phage required to show any visible clearing of cells when spotted in a 2 μl drop on a growing lawn of cells. LB broth (5 ml) containing 0.5 mM IPTG, ampicillin, chloramphenicol, and kanamycin was innoculated with single colony picks from the transductions listed above and incubated at 42°C. Following a 16 hr. incubation, the extent of growth in each culture was scored from no growth (0) to confluent (+++).
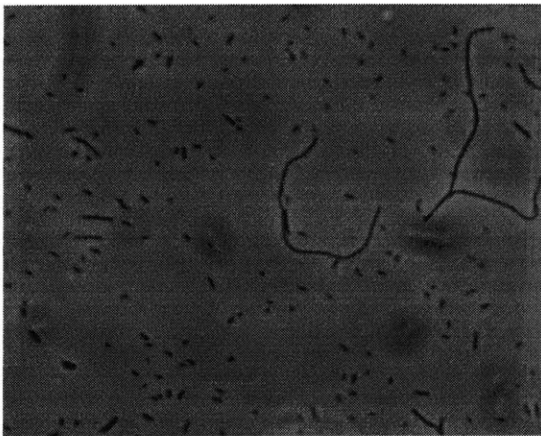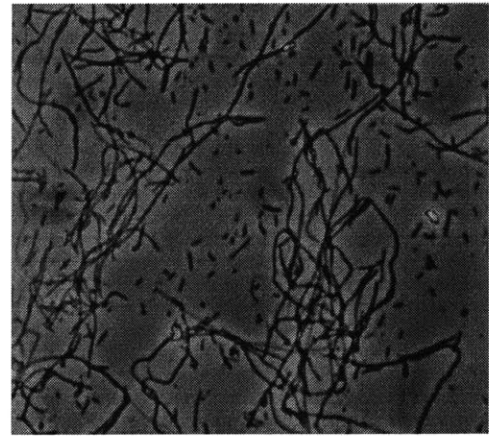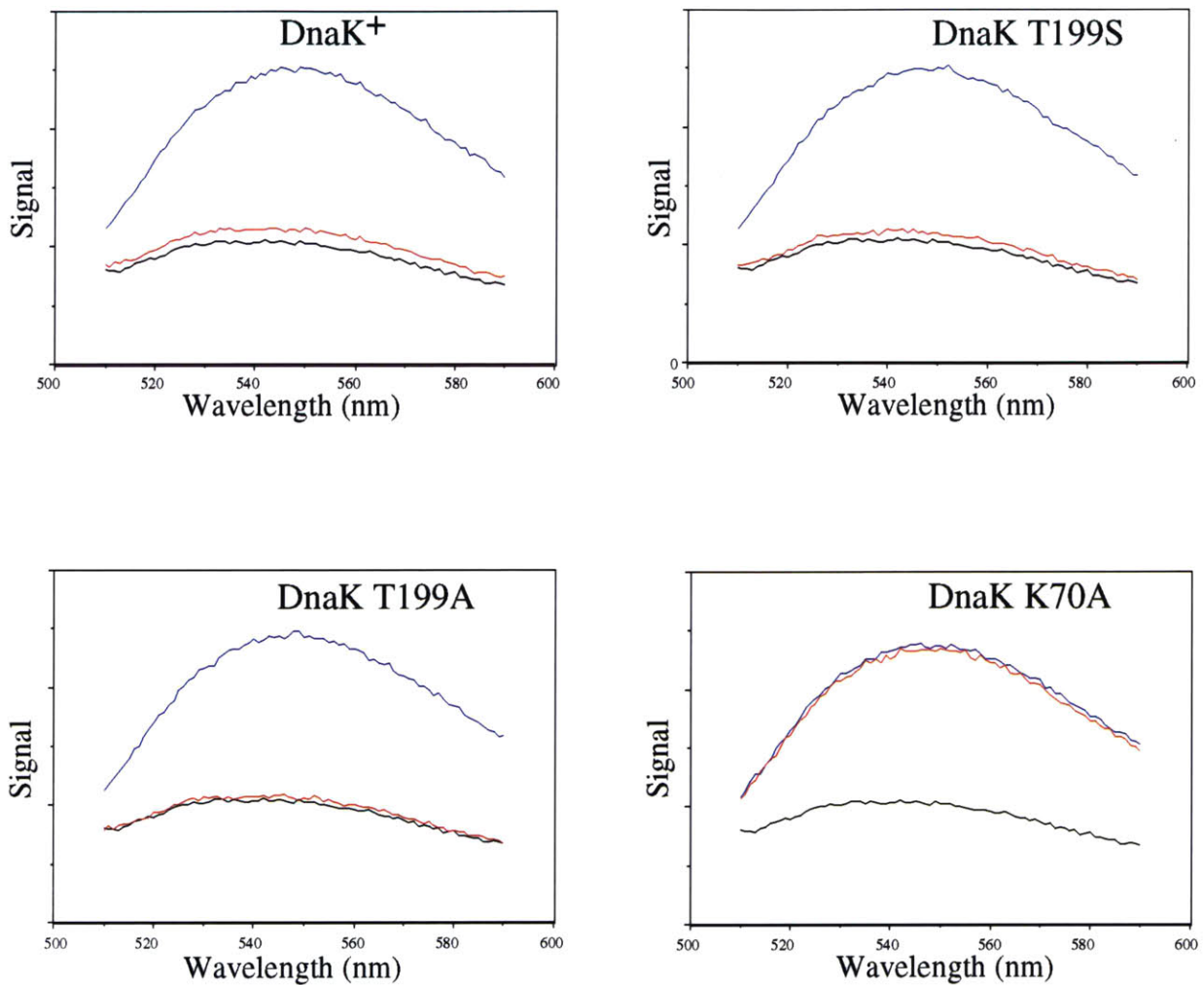
A.
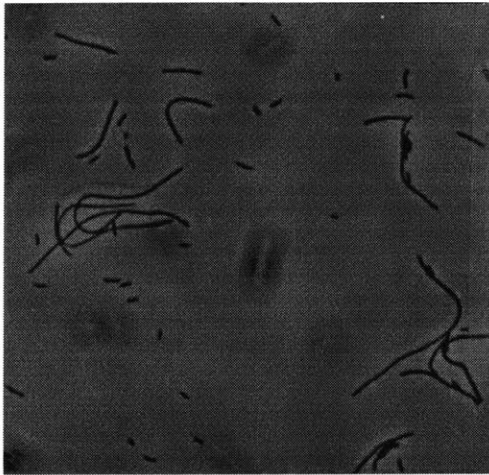
ΔdnaK52, pBR322
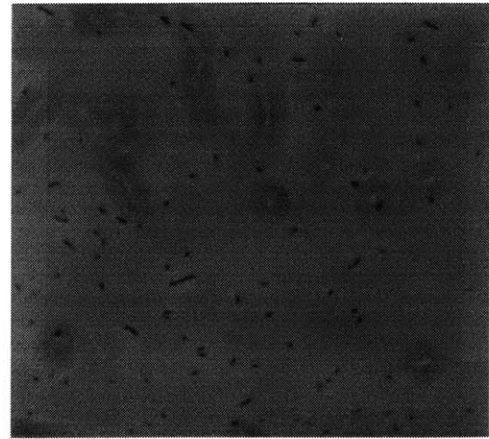
ΔdnaK52, pBR322(dnaK+)

ΔdnaK52, pBR322(dnaK(T199S))

ΔdnaK52, pBR322(dnaK(T199A))

B.

| DnaK	λ Sensitivity	Growth at 42˚C
wild type	1×10^6	+++
T199S	1×10^8	+
T199A	none	0
K70A	none	0

Figure 3. Peptide Binding and Release by DnaK and DnaK Mutants

DnaK or mutant DnaK (2.0 μM) was incubated with PepH-ANBD (1.0 μM) for 30 minutes
at 30°C in ATPase Buffer. The sample was split into two aliquots, and ATP (50 μM) was
added to one of the aliquots. The emission spectrum of each mixture was collected as described
in Materials and Methods. For comparision, the emission spectrum of PepH-ANBD (1.0 μM)
alone was also collected. Shown above are the spectra for DnaK and mutant DnaK where
the lines are: DnaK + PepH-ANBD (blue), DnaK + PepH-ANBD + ATP (red), and PepH-ANBD
alone (black).
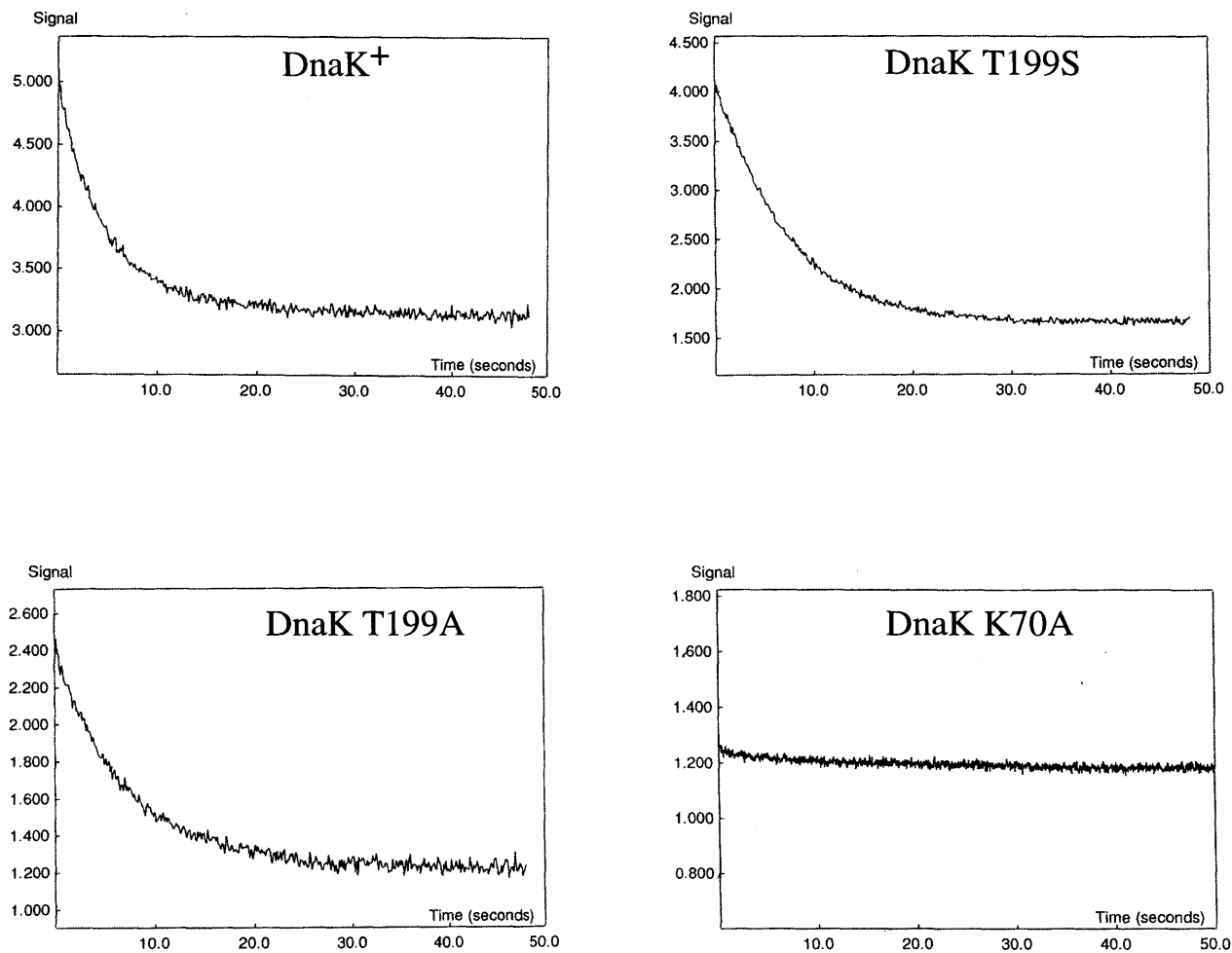
Figure 4. Kinetics of Peptide Release by DnaK and DnaK Mutants

DnaK or mutant DnaK (final concentration: 1 μM) was incubated with PepH-ANBD (final concentration: 0.5 μM) in ATPase Buffer for 30 minutes at 30°C and mixed in a stopped-flow apparatus with an equal volume of ATP (2 mM) in ATPase Buffer. The excitation wavelength used was 480 nm and a 495 nm cutoff filter was used. For details of fluorescence measurements, see Materials and Methods. All reaction traces, except for that of DnaK K70A, could be fit to a single-exponential decay function.
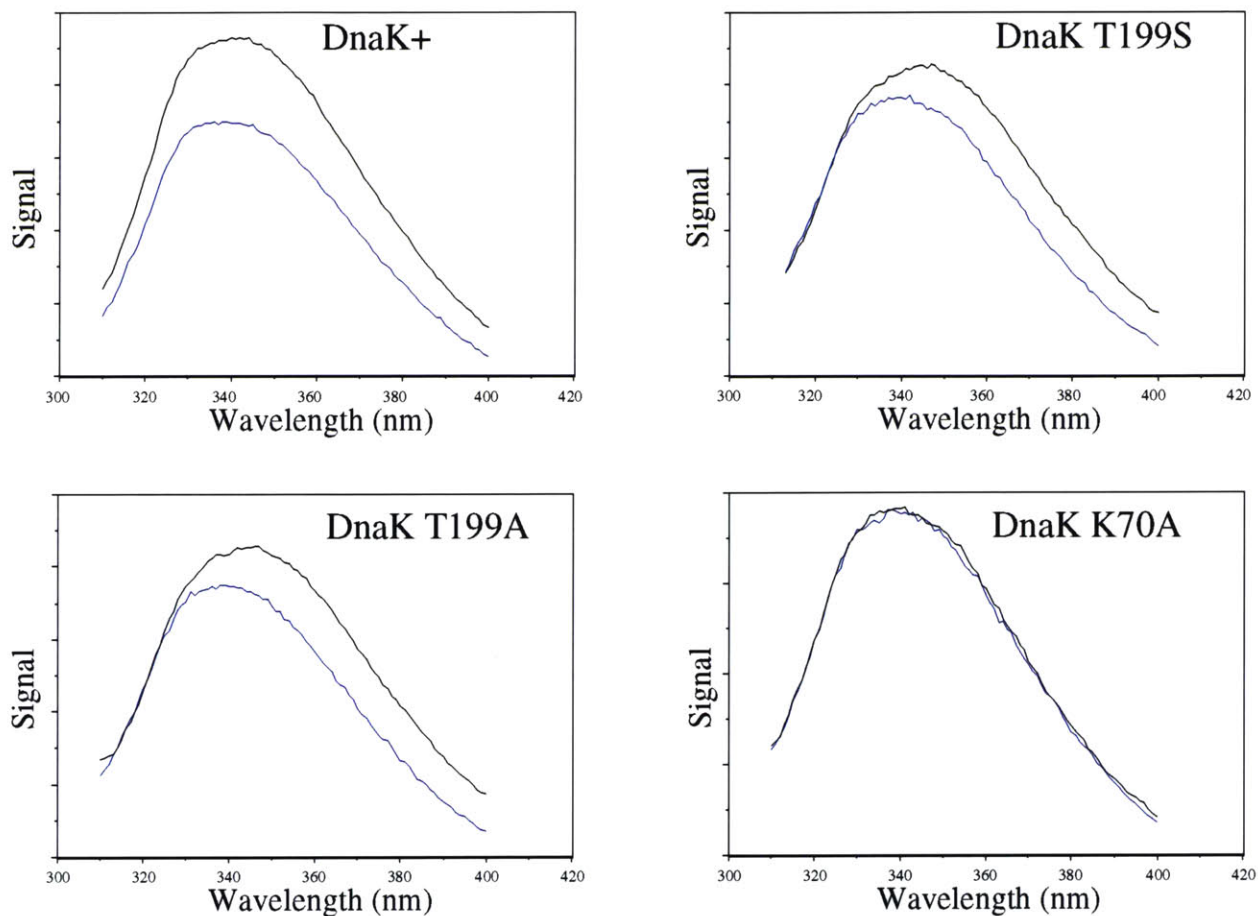
Figure 5. ATP Induced Conformational Change of DnaK and DnaK Mutants

The fluorescence of the single tryptophan residue in DnaK and mutant DnaK was measured in the absence (black line) or presence (blue line) of ATP. The fluorescence spectrum of DnaK and mutant DnaK (2.0 μM) with or without ATP (50 μM) in ATPase Buffer was collected as described in Materials and Methods. The spectrum of DnaK and the T199S and T199A mutants show a decrease in intensity and shift in the wavelength of maximal emission towards shorter wavelengths in the presence of ATP.

Chapter 5

A Novel Model for the Functional Cycle of DnaK:
Proposed, Retracted, and Revisited

Before we determined that the ADP kinase activity and the initial burst of ADP production in ATPase assays were the properties of a small amount of co-purifying NDP kinase in the preparations of DnaK and Hsp70 proteins, we constructed a novel model to describe the ATPase cycle of DnaK that accounted for these activities. A description of this model is presented below, followed by the experimental evidence that led to the model's construction. Following these descriptions is a discussion concerning aspects of this model that still provide an attractive alternative description of the ATPase cycle of DnaK, especially in light of our characterization of the DnaK K70A mutant. Finally, we discuss possible experimental approaches that could be used to test these possibilities.

The Original Model

A schematic depiction of the model described in the section is presented in Figure 1. DnaK hydrolyzes ATP rapidly and reversibly. Following the rapid binding of ATP to DnaK, the DnaK enters into a rapid equilibrium between two conformations, a low energy "DnaK" conformation and a high energy "DnaK*" conformation. The energy required to drive the conformational change from the DnaK form to the DnaK* form is provided by the hydrolysis of the γ-β phosphate bond of the bound ATP. Following the binding of ATP, the DnaK·nucleotide complex enters into a rapid equilibrium between DnaK·ATP and DnaK*·ADP·P_i. The energy of the γ-β phosphate bond of ATP is freely transferred between the DnaK protein to convert it into the DnaK* conformation and back into the nucleotide to rephosphorylate ADP·P_i to ATP. The phosphate in the DnaK*·ADP·P_i complex is either covalently bound to an amino acid residue of DnaK or noncovalently coordinated by interactions with amino acid residues of DnaK, ADP, and H_2O and K^+ and Mg^{++} ions inside the DnaK nucleotide binding pocket. The DnaK* form of DnaK represents a global conformational change from the DnaK form. A conformational shift in the amino-terminal ATPase domain triggered by ATP hydrolysis is coupled to a conformational change in the carboxyl-terminal peptide binding domain. This conformational change results in a lower affinity of the DnaK molecule for peptide, and is schematically depicted as the "lid open" state in

Figure 1. The opening of the peptide binding pocket results in an increase in the on-rate for peptide and an even larger increase in the off-rate, resulting in an overall lower affinity for peptide as previously described (17). The rapid equilibrium between DnaK·ATP and DnaK*·ADP·P$_i$ allows DnaK to scan the bound polypeptide by quickly converting between high peptide affinity and low peptide affinity forms. The ADP of the DnaK*·ADP·P$_i$ complex is rapidly exchangeable with the solvent and can be readily replaced by other ADP molecules in solution. Thus, the phosphorylation of ADP to ATP does not necessarily involve the same adenosine base that took part in hydrolysis, and gamma-phosphates are rapidly transferred between different adenosine bases.

The rate-limiting step of the cycle is the conversion of the DnaK* form to the DnaK form without the subsequent rephosphorylation of ADP. This step is depicted in Figure 1 as the DnaK*·ADP·P$_i$ -> DnaK·ADP·P$_i$ transition. Since the energy stored in the DnaK* conformation is lost, ADP cannot be rephosphorylated, and this step is irreversible. The rapid opening and closing of the peptide binding pocket stops, the peptide binding pocket remains closed, and a stable complex forms between DnaK and peptide. This rate-limiting step is stimulated by DnaJ and peptide sequences that bind to DnaK with high affinity. It is possible that these interactions stabilize the "lid closed" conformation of the binding pocket, and that the stabilization of this conformation is coupled to the stabilization of an ATPase domain conformation which favors the DnaK*·ADP·P$_i$ -> DnaK·ADP·P$_i$ transition.

ADP is bound in the DnaK·ADP·P$_i$ complex in a different manner than it is bound in the DnaK*·ADP·P$_i$ complex and is not freely exchangeable with the solvent. The release of ADP by DnaK·ADP·P$_i$ is relatively slow and becomes rate-limiting in the presence of DnaJ. The nucleotide exchange factor GrpE interacts with the amino-terminal ATPase domain of DnaK to open the nucleotide binding pocket and stimulate the rate of release of ADP. The nucleotide-free DnaK then binds ATP in two rapid steps: DnaK + ATP -> DnaK·ATP <-> DnaK*·ADP·P$_i$, the peptide is released, and the cycle repeats.

Building the Model

The observation of an initial burst of ADP production in ATPase assays led us to postulate that the hydrolysis of ATP by DnaK was a fast step in the ATPase cycle. The biphasic kinetics of ADP production which we observed (Chapter 2, Figure 1) are what one would expect to see with a cycle in which the steps leading to the first round of ADP production, the binding of ATP by DnaK and the hydrolysis of ATP to ADP and P_i, are not rate-limiting. These fast steps would be followed by a subsequent rate-limiting step, perhaps ADP release, which would be required to recycle DnaK and allow another round of hydrolysis. Thus, the first round of ADP production would be rapid, resulting in the stoichiometric initial burst, while the rate of all following rounds of ADP production would be governed by the rate-limiting step, resulting in the slow steady-state rate of hydrolysis.

At the time of our observations of the initial burst activity, the prevailing view in the literature was that the hydrolysis of ATP by DnaK was not reversible. We therefore reasoned that if hydrolysis was a fast step in the ATPase cycle of DnaK and some step following it was rate-limiting, then DnaK should spend more time with ADP bound than with ATP bound during steady-state hydrolysis, and more ADP should be bound to a population of DnaK than ATP. The observation that more ATP than ADP is bound to DnaK during steady-state hydrolysis (Chapter 2, Figure 2) was consistent with previous reports (5, 9) and led us to postulate that the hydrolysis of ATP was reversible. The ATP:ADP ratio could represent an equilibrium, DnaK·ATP <-> DnaK*·ADP·P_i, with the form on the left being predominant. Our observation that the addition of ADP to ATPase assays increased the magnitude of the initial burst (Chapter 1, Figure 3) led us to propose that the DnaK*·ADP·P_i complex can rapidly and reversibly release ADP. Thus a reaction pathway DnaK + ATP <-> DnaK·ATP <-> DnaK*·ADP·P_i <-> DnaK*·P_i + ADP was proposed. This reaction pathway predicted that DnaK had an ADP kinase activity, the ability to phosphorylate ADP to ATP in the presence of ATP. This activity would result in the equal sharing of all gamma-phosphates by all adenosine nucleotides in a reaction mixture. Thus, if 2 μM of unlabeled ATP were added to 1 μM [^{14}C]ADP, the 2 μM of gamma-phosphate would be equally distributed

among the 3 μM of adenosine, and the ratio of $[^{14}C]$ATP to $[^{14}C]$ADP would be 2 to 1. We did measure an ADP kinase activity in our DnaK preparations (Chapter 2, Figure 5). Complete redistribution of gamma-phosphate between 20 μM ATP and 61.2 μM $[^{14}C]$ADP would result in a $[^{14}C]$ATP/($[^{14}C]$ATP + $[^{14}C]$ADP) ratio of 0.246, very similar to the value that we observed following a two minute incubation of DnaK with these nucleotide concentrations. This result implied an ADP kinase activity by DnaK which was much faster than its steady-state ATPase rate.

The model could be used to explain the results of other researchers who had used their results to support a alternative model of the functional cycle of DnaK. Anthony Fink's group concluded that ATP hydrolysis is not required to elicit peptide release by DnaK in part because the DnaK T199A mutant, which has a defective ATPase activity (10), releases peptide when mixed with ATP (13). This group assumed that the defective step in the ATPase cycle of the DnaK T199A mutant was ATP hydrolysis itself. We proposed that DnaK T199A could be defective in a different step in the ATPase cycle, such as the DnaK*·ADP·P$_i$ -> DnaK·ADP·P$_i$ transition, and the hydrolysis of ATP by DnaK T199A could still be intact and result in peptide release. Other researchers had concluded that since the majority of nucleotide bound to DnaK during steady-state hydrolysis of ATP is ATP, hydrolysis must be the rate-limiting step of the ATPase cycle (5, 9). This conclusion was based on the assumption that ATP hydrolysis is non-reversible. Our model stated that the bound ATP to ADP ratio resulted from the DnaK·ATP <-> DnaK*·ADP·P$_i$ equilibrium resulting from reversible hydrolysis of ATP.

The results shown in Chapter 2, Figures 6 and 7 were not entirely consistent with our model, but they could be explained by making minor revisions to our model. The ability of the DnaK preparations to phosphorylate CDP as well as ADP (Chapter 2, Figure 6) could still be due to an activity intrinsic to DnaK if DnaK were able to phosphorylate a range of NDP substrates, similar to the manner in which DnaK had been reported to hydrolyze a range of NTP substrates (8). The conclusion that the initial burst of $[\alpha-^{32}P]$ADP production in ATPase assays was a result of the phosphorylation of unlabeled ADP to unlabeled ATP with the gamma-phosphate of $[\alpha-^{32}P]$ATP (Chapter 2, Figure 7) could be explained if the DnaK·ATP <-> DnaK*·ADP·P$_i$

equilibrium lay far to the left, so that no true initial burst of ADP was produced. The DnaK could release its bound ADP and phosphorylate it with the gamma-phosphate of [α-^{32}P]ATP, resulting in the burst of [α-^{32}P]ADP. However, the observation that the ADP kinase activity in the *Thermus thermophilus* DnaK 44-kDa amino-terminal fragment preparation was abolished at high temperatures together with the observation that the amount of ADP kinase activity in DnaK preparations purified from *ndk::km* cells was much less than the amount of ADP kinase activity in DnaK preparations purified from *ndk*⁺ cells, led us to the conclusion that DnaK does not have an intrinsic ADP kinase activity. Thus our model as originally formulated was determined to be invalid.

Is ATP Hydrolysis by DnaK Required for Conformational Change and Peptide Release?

The finding that the DnaK K70A mutant does not undergo a conformational change (Chapter 4, Figure 5) or release peptide (Chapter 4, Figure 3 and Figure 4) when mixed with ATP has led us to consider one central aspect of our original model for the functional cycle of DnaK. The proposal that the ε-amino side chain of lysine-71 of the Hsc70 protein stabilizes an H_2O molecule or OH^- ion for a nucleophilic attack on the γ-phosphate of ATP (4, 19) was confirmed by structural and kinetic studies of lysine-71 mutants of the 44-kDa amino-terminal ATPase fragment of bovine Hsc70 (11). These studies implicate lysine-71 as an indispensable residue for the hydrolysis of the γ-β phosphate bond of ATP by Hsc70. Since this residue is completely conserved in the Hsp70 family, and since the crystal structures of the ATPase domain of Hsc70 (3) and DnaK (7) are nearly identical, it would be surprising if the lysine-70 residue of DnaK did not play the same role that lysine-71 plays in Hsc70. Substitution of lysine-70 of DnaK with alanine resulted in a greatly reduced ATPase activity (Chapter 4, Figure 1), similar to the findings with Hsc70 lysine-71 mutants (11). It is therefore significant that a DnaK mutant that is presumed to be unable to hydrolyze ATP is unable to undergo an ATP induced conformational change. It should be noted that the DnaK T199A mutant, used to support the hypothesis that ATP hydrolysis is not

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required for the ATP induced conformational change (13) has a defective ATPase activity [(10, 13), (this study, Chapter 4, Figure 1)], but is probably able to hydrolyze ATP as the homologous threonine-204 residue of Hsc70 was found not to be essential for hydrolysis based upon structural studies of the ATPase domain of Hsc70 (12). Thus, the notion that ATP hydrolysis results in the ATP induced conformational change of Hsp70 proteins still seems to us to be viable. Since the conformational change occurs much more quickly than the products of ATP hydrolysis are formed, we believe that it is possible that the hydrolysis of ATP by DnaK is rapid and reversible, similar to the rapid, reversible hydrolysis of ATP by molecular motor proteins such as myosin (16) and kinesin (6). While DnaK does not possess an ADP kinase activity, we believe that one aspect of our old model, the rapid DnaK·ATP <-> DnaK*·ADP·P$_i$ cannot yet be discarded.

The notion that ATP hydrolysis by DnaK is rapid and reversible and is coupled to the conformational change that results in peptide release is appealing for several reasons. DnaK does not undergo a conformational change or release bound peptide when mixed with any of the nonhydrolyzable analogs of ATP, AMP-PNP, AMP-PCP, or ATPγS (8, 14, 17). Several groups have concluded that the binding of ATP alone results in the conformational change in DnaK that causes peptide release (1, 2, 13, 15, 18). However, all of the nonhydrolyzable analogs of ATP act as competitive inhibitors of ATP, and the crystal structure of AMP-PNP complexed with the ATPase fragment of Hsc70 was determined to a resolution of 2.4 Å and found to resemble the structure of Hsc70 complexed with ADP + P$_i$ so closely that it was used as a model for the prehydrolysis binding of ATP to Hsc70 (4). Thus, it seems more likely that nonhydrolyzable analogs of ATP do not induce a conformational change in DnaK because ATP hydrolysis is required for the conformational change, than that they do not bind DnaK with the correct geometry to induce a conformational change. Also, ATP has been observed to bind to DnaK in two steps, a rapid initial binding step followed by a slower step that coincides with the conformational change of DnaK and peptide release (18). The DnaK·ATP <-> DnaK*·ADP·P$_i$ equilibrium state could provide a structural explanation for this second step of ATP binding. Finally, based on structural observations, residue threonine-204 of the ATPase fragment of Hsc70, and presumably threonine-

199 of DnaK, is not essential for the hydrolysis of ATP (12). However, certain substitutions of threonine-199 in DnaK result in proteins with greatly reduced ATPase activities. The DnaK·ATP <-> DnaK*·ADP·P$_i$ equilibrium would be part of a pathway that requires other steps in order to produce the end products of ATP hydrolysis, free ADP and free P$_i$. The threonine-199 mutants of DnaK could be defective in one of these steps, and thus would be able to hydrolyze the γ-β phosphate bond of ATP but still have a ATPase activity with a lowered rate. A rate-limiting step would be required following the DnaK·ATP <-> DnaK*·ADP·P$_i$ state, but prior to the DnaK·ADP -> DnaK + ADP step to account for the low steady-state rate of ATP hydrolysis by DnaK. This step could be the DnaK* -> DnaK transition, similar to our original model. This step could be defective in threonine-199 mutants of DnaK. The conserved, autophosphorylated residue threonine-199 of DnaK is interesting, because it is completely conserved in the Hsp70 family and even a conservative substitution of the residue, the T199S mutant in this study, results in a protein that is unable to fully function as DnaK. We found that DnaK T199S has a near normal ATPase activity and undergoes a ATP induced conformational change, but is not able to fully function as DnaK in the cell. These results suggest that residue threonine-199 is required for some step other than ATP hydrolysis, but which is still required for proper functioning, in the ATPase cycle of DnaK.

The existence of a rapid DnaK·ATP <-> DnaK*·ADP·P$_i$ equilibrium could be difficult to prove experimentally. Positional isotope exchange experiments could be used in which DnaK would be incubated with ATP and ^{18}O labeled H$_2$O. The ^{18}O label could also be placed on the β-phosphate of the ATP. The incorporation of multiple ^{18}O isotopes per single γ-phosphate of ATP would provide evidence for rapid, reversible hydrolysis of ATP by DnaK. The rapid appearance of one or more ^{18}O isotopes per γ-phosphate of ATP in chemical quenched-flow experiments would also support the existence of rapid, reversible hydrolysis of ATP by DnaK. However, if DnaK does carry out rapid, reversible hydrolysis of ATP, but there is no free rotation of the phosphate in the DnaK*·ADP·P$_i$ state, then positional isotope exchange experiments would not

yield positive results. It may require the kinetic and structural characterization of more DnaK

mutants to make a final and definitive description of the functional cycle of DnaK.

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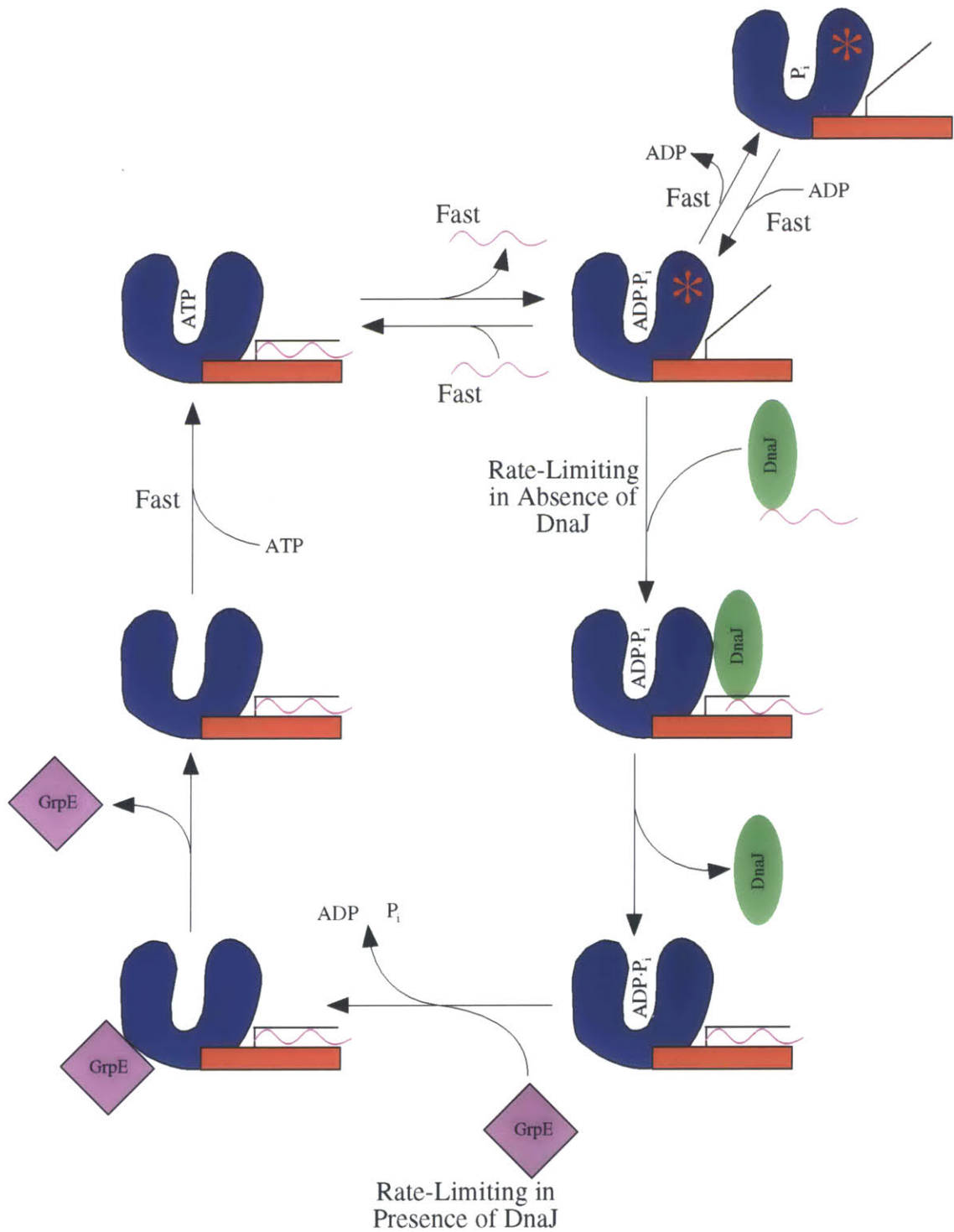
Figure 1. Model for the Functional Cycle of DnaK

DnaK is represented by the object: ◡ (low energy, high peptide affinity) and

◡ (high energy, low peptide affinity), where blue is the ATPase domain and red is the peptide binding domain. See text for details.