Inferences Concerning the ATPase Properties of DnaK and Other HSP70s Are Affected by the ADP Kinase Activity of Copurifying Nucleoside-diphosphate Kinase*

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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 $[^{32}P]ATP$ when incubated with $[^{32}P]ADP$ and contain an ADP kinase activity. We determined that the initial burst activity results from the transfer of $\gamma$-phosphate from the radiolabeled substrate $[^{32}P]ATP$ to unlabeled ADP bound by DnaK and is the same activity that results in ADP phosphorylation. 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 with 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 isolated from ndk+ cells. The remaining ADP kinase activity in preparations from ndk::km cells, which varies between preparations, is also a property of NDP kinase, which is most likely expressed because of a low frequency reversion of the disrupted 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. The presence of contaminating NDP kinase can explain the range of $k_{cat}$ values reported for the ATPase activity of DnaK as well as recent reports of initial burst kinetics by DnaK (Banacki, B., and Zylicz, M. (1996) J. Biol. Chem. 271, 6137–6143) and an ADP-ATP exchange activity of DnaK (Hiro-mura, M., Yano, M., Mori, H., Inoue, M., and Kido, H. (1998) J. Biol. Chem. 273, 5435–5438).

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 (3, 4), intracellular protein trafficking, membrane translocation (5), protein degradation (6), and disassembly of native protein complexes (7). HSP70 proteins function as molecular chaperones in all of these processes, undergoing multiple cycles of binding and release of the polypeptide substrate to facilitate its folding or to protect unfolded states from aggregation and denaturation (8–12). DnaK acts as a molecular chaperone in E. coli. In vitro, DnaK blocks the aggregation and allows the reactivation of heat-inactivated RNA polymerase (13), rhodanese (14), and firefly luciferase (15). DnaK dissociates dimeric RepA protein to allow P1 phage replication (16) and promotes the disassembly of the protein complex at ori-λ, allowing replication (17). DnaK is required for growth at high temperature (18) and is required for the negative regulation of the heat shock response at normal growth temperatures (19). DnaK represses the expression of heat shock genes by negatively regulating the synthesis and stability of $\sigma^{32}$ (6, 20), and this repression is transiently lifted upon heat shock (21).

Limited proteolysis of HSP70 proteins separates them into two stable domains. The COOH-terminal domain binds peptides in an extended conformation (22, 23) in a manner believed to mimic the binding of physiological substrates. The NH$_2$-terminal domain possesses the weak intrinsic ATPase activity associated with every HSP70 protein (24, 25). The two domains interact, and the peptide binding and release cycle is tightly coupled to the ATP binding and hydrolysis cycle (8, 9, 11, 26). 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 has been reported. The ATPase activity of unstimulated DnaK has been assigned $k_{cat}$ values ranging from 0.02 to 1.0 min$^{-1}$ (27–29) and $K_m$ values ranging from 20 nM to 4 μM (30, 31). 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 (32), which initially led to the conclusion that ATP hydrolysis results in a global conformational change of DnaK that in turn results in peptide release. However, because mutants of DnaK that have a defective ATPase activity release peptide (33) and undergo a conformational change (34) upon ATP binding, it was concluded that ATP binding, and not hydrolysis, causes a conformational change of DnaK that results in peptide release. Measurements of the amounts of ATP and ADP bound to DnaK during ATP hydrolysis (35) and of single ATP turnover rates (36, 37) 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 (29, 37–39).

We found that a variety of DnaK and HSP70 preparations display initial burst kinetics in ATPase assays carried out with $[^{32}P]ATP$, rapidly producing a stoichiometrically equal amount of $[^{32}P]ADP$ when incubated with ATP. We also found that these preparations contain an ADP kinase activity, phosphorylating ADP to ATP in the presence of ATP. However,
brane molecular weight cut-off = 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 (Amersham Pharmacia Biotech) column (250 ml) and resolved by running a linear 50 mM-550 mM KC1 gradient over 500 ml in Buffer B. The total concentration of ADP was recalculated following the addition of the radiolabeled ADP. Unless otherwise indicated, the ATP and ADPase Buffer were mixed and preincubated at 30 °C for 5 min, and hydrolysis was initiated by the addition of DnaK, marked as time 0 in the assays. When present in the reaction mixture, ADP and ovabumin 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 polyethyleneimine-cellulose TLC plate (J. T. Baker Inc.,Phillipsburg, NJ). Spotting the reaction 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 a 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 PhosphorImage 445 Si and analyzed using the PhosphorImage QuantiSoft program. The amount of each species present was determined by volume integration. The data were corrected for the level of background hydrolysis (typically 1% or less). The extent of hydrolysis was expressed as the fraction of ADP or P, to total nucleotide, or further calculations were made to determine the velocity of the reaction. The velocity of the ATPase reaction in terms of pmol of ATP hydrolyzed/min was determined by multiplying the measured (A$_{280}$) + P) 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**—Reaction mixtures (25 ml) contained ATPase Buffer, [8-14C]ADP, ATP, and DnaK. Radiolabeled ATP stocks were made by adding radiolabeled ATP to unlabeled ATP to give a final activity of 4.00 mCi/ml. The total concentration of ADP was recalculated following the addition of the radiolabeled ATP. The ATP, ADP, and ATPase Buffer were mixed and preincubated at 30 °C for 5 min, 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 [2-14C]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 polyethyleneimine-cellulose TLC plate. The TLC plate was developed and analyzed exactly as for the ATPase assays described above. The 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. The velocity of the ADP kinase reaction in terms of pmol of ADP phosphorylated/min was determined by multiplying the measured (A$_{280}$ + ADP) ratio by the starting amount of ADP in the reaction (indicated in the corresponding figure for each reaction) and dividing by time.

**Nucleotide Removal Treatment**—Nucleotide was removed from DnaK by a method similar to that previously described for Hsc70 (49). DnaK

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; AMP-PNP, adenosine 5′-O-(3-acetonitrilophosphoribosyl) AMP; IMAC, immobilized metal-ion affinity chromatography; NDP, nucleoside-diphosphate kinase.
was saturated with AMP-PNP (5 mM) in a total volume of 2 ml and incubated at 25 °C for 1 h. To remove free AMP-PNP, the sample was applied to a G-25M desalting column (Amersham Pharmacia Biotech PD-10 column) equilibrated with Buffer B and eluted with 3.5 ml of Buffer B. The sample was dialyzed in a Spectra/Por Membrane molecular weight cut-off = 12,000–14,000 Da against Buffer B (700 ml) three times. The dialed sample was applied to a MonoQ column and eluted with a double linear gradient of 50–225 mM KCl over 7.5 ml followed by 225–435 mM KCl over 45 ml in Buffer B.

**Production and Purification of His-tagged Nucleoside-diphosphate Kinase (NDP Kinase)—** A plasmid was constructed to express NDP kinase with an amino-terminal tag of 10 histidine residues. Two-mL primers were purchased from Life Technologies, Inc. designed to amplify the ndk gene from genomic E. coli DNA based on the known sequence of the gene (50) and include a 5'-NdeI and a 3'-BamHI restriction site. The NdeI-ndk-BamHI product was amplified by polymerase chain reaction using the GeneAmp kit (Perkin-Elmer Corp.). The amplification product was purified by subjecting it to agarose gel electrophoresis and by extracting its band using a QIAquick polymerase chain reaction 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 (A600 ~ 0.6) and induced with 0.4 mM isopropyl-1-thio-β-galactosidase to overexpress His-tagged NDP kinase (Ndk-N-His). Following incubation at 37 °C for 1 h, the cells were pelleted by centrifugation (5000 × g, 20 min, 4 °C), resuspended in 1× 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 (30,000 rpm, 20 min, 4 °C), and the supernatant was applied to a 2.5 ml His-Bind immobilized metal-ion affinity chromatography (IMAC) column (Novagen) equilibrated with 1× binding buffer, low salt. For co-purification experiments, the column was washed with 10 ml of 1× binding buffer, low salt followed by 10 ml of 0.5× wash buffer, low salt (30 mM imidazole, 50 mM NaCl, 20 mM Tris-HCl, pH 7.9). The gel bed was then resuspended in 0.5× wash buffer, low salt, and the gel suspension was transferred into an Amersham Pharmacia Biotech XK16 column. The XK16 column was attached to a Amersham Pharmacia Biotech fast protein liquid chromatography system, and the gel bed was settled with 0.5× wash buffer, low salt at a flow rate of 0.5 ml/min. A linear gradient of 30–500 mM imidazole was then run over 30 ml in 0.5× 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 (40), incubated in a boiling water bath for 3 min, and subjected to SDS-PAGE as described (40). The proteins were transferred from the gels to polyvinylidene difluoride 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).

**Protein Sequencing—** Protein sequencing was performed at the M.I.T. Polymers Laboratory. The sample was loaded onto an Applied Biosystems PreSorb sample preparation cartridge with a polyvinylidenefluoride membrane. The sample was then sequenced with an Applied Biosystems Procise 494 protein sequencer.

**RESULTS**

**Preparations of DnaK and Other HIS70 Proteins Hydrolyze (α,β-32P)ATP with Biphasic Kinetics—** In the experiments described below, we used DnaK that we had purified from DnaK-overproducing E. coli cells by a method that involves three primary chromatographic steps: DEAE-Sepharose anion exchange, ATP-agarose affinity, and MonoQ anion exchange column. This method is basically the same one used by other groups in the field (e.g. 35, 44, 45). 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,β-32P)ADP production appeared constant over time when the molar amount of (α-32P)ATP in the assay was 100-fold or more greater than the molar amount of DnaK. However, when the molar amount of (α-32P)ATP was less than 100-fold greater than the molar amount of DnaK, an initial burst of (α-32P)ADP formation became apparent. When the amount of (α-32P)ATP was only severalfold greater than the amount of DnaK in the assay, the rate of (α-32P)ADP formation clearly showed biphasic kinetics: an initial burst of (α-32P)ADP formation within the first min followed by a much slower steady-state rate of ADP formation. (α-32P)ATP was incubated with several different concentrations of DnaK at 30 °C, and the amount of ADP produced was measured over time (Fig. 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 Fig. 1A, the sizes 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. Because 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 μM of ADP for 1.44 μM, 2.88 μM, and 5.77 μM of DnaK, respectively. 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 because of the loss of DnaK from solution, and the addition of 2.0 μg/μl ovalbumin to the assay buffer to act as a nonspecific carrier for DnaK corrected this problem. The addition of ovalbumin to assays with DnaK present at micromolar concentrations had little effect on the kinetic profile of the ATPase reaction (Fig. 1B), and therefore there was no significant loss of DnaK from solution at these concentrations. DnaK was also preincubated at 30 °C for 30 min prior.
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 ATP formation was a true characteristic of our DnaK preparations and was not because of any loss of enzymatic activity.

Several different preparations of DnaK from our lab were similarly assayed, and all showed a stoichiometric burst of \( [\alpha-^{32}P]ADP \) production. 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 \( T. \) 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 (data not shown).

DnaK Preparations Phosphorylate ADP to ATP in the Presence of ATP—We obtained results not described here that led us to consider the possibility that the DnaK samples we were analyzing might be able to phosphorylate ADP in the presence of ATP. DnaK was incubated with ATP and \([8-^{14}C]ADP\) at 30 °C, and the radiolabeled nucleotide content of the mixture was analyzed at various times. Fig. 2A 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. 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 because of the steady-state hydrolysis of \([8-^{14}C]ATP\) to \([8-^{14}C]ADP\) by DnaK.

The same DnaK, HSP70, Hsc70, and BiP preparations discussed previously were assayed. DnaK preparations from three different labs, the amino-terminal ATPase fragment of DnaK from \( T. \) 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).

NDP Kinase Affects the ATPase Activity of DnaK Preparations

ADP Kinase and Initial Burst Activities of DnaK/HSP70 Preparations Are because of 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 that we had assayed were because of 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 \( \mu \)g of DnaK (lane (data not shown). Because Coomassie Brilliant Blue staining allows the detection of as little as 0.1 \( \mu \)g of protein in a single band (40), 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 NDP kinase in preparations of some proteins had resulted in a flawed analysis of the ATPase activity of the protein.2 NDP kinase catalyzes the transfer of \( \gamma \)-phosphate from a nucleoside triphosphate to a nucleoside diphosphate with a turnover rate of approximately 1000 \( s^{-1} \) under conditions similar to those used in our ATPase assays (51). This turnover rate is 600,000–6,000,000 times higher than most published values of the turn-over rate of the ATPase activity of DnaK, and therefore a relatively tiny amount of NDP kinase could significantly affect the kinetic properties of the ATPase activity of a preparation of DnaK. Thus we were concerned 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.

NDP kinase is substrate-nonspecific and catalyzes the transfer of phosphate from any nucleoside triphosphate to any nucleoside diphosphate with nearly equal efficiency (2). DnaK, on the other hand, while reported to hydrolyze GTP, CTP, and UTP to some extent, hydrolyzes ATP with the greatest efficiency (32), and ATP is believed to be required for proper DnaK function (32, 33). 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 because of some other protein. All DnaK and Hsc70 preparations discussed above were therefore incubated with ATP and either radiolabeled ADP or radiolabeled CDP. When radiolabeled ADP was provided as a phosphate acceptor, radiolabeled ATP was produced, whereas when radiolabeled CDP was provided as a phosphate acceptor, radiolabeled CTP was produced with similar kinetics (data not shown). Thus, the DnaK and HSP70 preparations were nonspecific 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 (49). 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 therefore likely to have some ADP bound. Thus, NDP kinase would be able to use this HSP70-bound ADP as a phos-

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2. B. Bukau, personal communication.
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Fig. 3. Burst of \( [\alpha-^{32}P]ADP \) produced by DnaK preparations is because of phosphorylation of bound ADP. A, DnaK (2.89 \( \mu \text{M} \)) was added to either \( [\alpha-^{32}P]ATP \) or \( [\gamma-^{32}P]ATP \) (19.8 \( \mu \text{M} \)) with or without ADP (20 \( \mu \text{M} \)) in ATPase Buffer and incubated at 30 °C. The reaction was stopped following various incubation times, and the amount of radiolabeled ATP and radiolabeled ADP or radiolabeled P was determined. The results were plotted as follows: 1. \( [\alpha-^{32}P]ATP \); 2. \( [\alpha-^{32}P]ATP + ADP \); 3. \( [\gamma-^{32}P]ATP \); 4. \( [\gamma-^{32}P]ATP + ADP \). B, DnaK (3.01 \( \mu \text{M} \)) was incubated with a saturating concentration of ATP (20 \( \mu \text{m} \)) in ATPase Buffer at 30 °C for 10 min. Free nucleotide was removed from the DnaK by applying the sample to a Bio-Rad BioSpin 30 column. DnaK saturated with ATP (●) or not saturated with ATP (○) was added to \( [\alpha-^{32}P]ATP \) in ATPase Buffer and incubated at 30 °C. The reaction was stopped following various incubation times, and the amount of ADP and ATP was determined.

and a burst of \( [\gamma-^{32}P]ADP \) formation that 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 \( [\gamma-^{32}P]ATP \), which is 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 \( [\alpha-^{32}P]P \), which would terminate the reaction when the ADP kinase activity in the DnaK preparation is a property of a 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. thermophilus DnaK 44-kDa NH2-terminal preparation were measured over a temperature range of 30–90 °C. Whereas 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, although the ATPase activity was very robust at this temperature (data not shown). 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 that is present at very low levels in the preparations.

Purification of DnaK from ndk::km E. coli Cells Removes Most, but Not All, of the ADP Kinase Activity in DnaK Preparations—The observations 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 because of the transfer of phosphate from \( [\alpha-^{32}P]ATP \) to ADP, and that the ADP kinase activity in T. thermophilus DnaK preparations is inactivated at high temperature led us 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 NusA/nucleoside-diphosphate kinase family copurifies with Hsc70 (52) lent support to the hypothesis that the protein responsible for the ADP kinase activity in the DnaK and HSP70 preparations that we analyzed is NDP kinase. To test this hypothesis, DnaK was purified from E. coli cells with a disruption in the ndk gene. A ndk::km DnaK strain was constructed by transducing the \( \Delta dnaK52 \) allele into the E. coli strain NA7623. The ndk gene of NA7623 is disrupted by a kanamycin-resistance gene insert (41). The isogenic ndk::km DnaK strain was constructed by transducing the \( \Delta dnaK52 \) allele into the ndk+ parent strain of NA7623,
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Massie Brilliant Blue-stained SDS-PAGE gels of the MonoQ A

were treated to remove bound ADP by extensive dialysis as described previously (49). When the nucleotide-free DnaK was re-injected onto a MonoQ column, it eluted as one major peak followed by several much smaller broad peaks (Fig. 5B). 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 Fig. 5B and saturated with 2 mM ATP. Following a 15-min incubation at 25 °C, the DnaK was immediately loaded onto a MonoQ column. The DnaK does not separate into multiple peaks following saturation with ATP (data not shown), 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 that converts the DnaK from several forms into one predominant form. One possible explanation for this conversion is that the treatment removes peptide fragments that are bound by DnaK.

ADP phosphorylation assays were performed on the MonoQ fractions of DnaK preceding and following treatment to remove bound nucleotide. MonoQ fraction 13 of the nucleotide-free DnaK preparation, which contains very little DnaK, has the highest ADP kinase activity of all the MonoQ fractions (Fig. 5B). 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 (Fig. 5, A and B). The ADP kinase activity in MonoQ fraction 15, which contains the highest amount of nucleotide-free DnaK, is less than 0.2 pmol of ADP phosphorylated/min/5 μl, nearly 50-fold lower than the 8.98 pmol of ADP phosphorylated/min/5 μl for the MonoQ fraction containing the highest amount of untreated DnaK (Fig. 5, A and B).

To confirm that nucleoside-diphosphate kinase is responsible for the initial burst of [α-32P]ADP produced when certain DnaK preparations are incubated with [α-32P]ATP, the rate of [α-32P]ADP produced by single peak, nucleotide-free DnaK prepared from ndk::km cells (fraction 15 in Fig. 5B) incubated with [α-32P]ATP was compared with the rate of [α-32P]ADP produced by DnaK prepared from ndk+ cells incubated with

![Figure 4](https://example.com/figure4.png)

**FIG. 4.** Chromatograms of protein concentration and ADP kinase activity from MonoQ separation of DnaK. DnaK overexpressed in either ndk− (A) or ndk::km (B) 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 the scales of the right y axis between the two panels.

![Figure 5](https://example.com/figure5.png)

**FIG. 5.** MonoQ chromatograms of DnaK separation with and without nucleotide-free treatment. A, Mono Q chromatogram of DnaK without treatment to remove nucleotide (left panel). The chromatogram shows a trace 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). Fractions from the MonoQ separation were collected and assayed for total protein concentration (□) and ADP kinase activity (●) (right panel). B, Mono Q chromatogram and ADP kinase activity of DnaK following treatment to remove nucleotide. Data are presented as for A.
DnaK preparations 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 pmol of ADP phosphorylated/min/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 strains. Following the treatment to remove nucleotide from the DnaK and MonoQ chromatography, the MonoQ fraction with the highest level of ADP kinase activity was free of DnaK. The fraction with the highest amount of ADP kinase activity, MonoQ fraction 13, phosphorylated ADP at a rate of 15,000 pmol/min/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 affinity chromatography to look for evidence of a physical interaction between the two proteins. The ndk gene was cloned by polymerase chain reaction 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 Ni2+ ions and can be purified in a single step by IMAC. 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 30–500 mM imidazole gradient. Shown are Coomassie Brilliant Blue-stained SDS-PAGE gels (top) and Western blots of SDS-PAGE gels identical to the Coomassie Brilliant Blue-stained gels above probed with α-DnaK antibody (bottom). The lanes are labeled as follows: S, molecular mass standards; C, crude lysate supernatant; F, IMAC flow through; W, IMAC wash; numbers, eluent fraction number (fraction 1–30 m m imidazole to fraction 23–390 m m imidazole over a linear gradient).
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 (Fig. 7). 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 because of 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.

**DISCUSSION**

Preparations of *E. coli* DnaK from our lab, which have been purified by a procedure very similar to those employed by numerous other labs, were found to contain an ADP kinase activity that is because of the presence of small amounts of co-purifying NDP kinase. The NDP kinase that 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 several other labs, the 44-kDa amino-terminal ATPase domain of *T. 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. DnaK purified from *E. coli* cells containing a disruption of the *ndk* gene typically has approximately a 500-fold reduction in the level of co-purifying ADP kinase activity when compared with DnaK purified from *ndk* + cells. This observation confirms that the ADP kinase activity found in preparations of DnaK is because of the presence of a very small amount of co-purifying NDP kinase.

The implications of the presence of NDP kinase in DnaK preparations are 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 γ-phosphate transfer activity, has an ATPase activity with a *k*₅₀ value much higher than that of DnaK (51). The rapid kinetics of ATP hydrolysis by NDP kinase makes accurate kinetic measurements of DnaK ATPase impossible in its presence. The presence of differing amounts of undetected NDP kinase in DnaK preparations from different labs could explain, at least in part, the wide range of *k*₅₀ values reported for the ATPase activity of DnaK from 0.018 min⁻¹ (29) to 0.13 min⁻¹ (54, 55) to 1.0 min⁻¹ (28). 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 (36).² NDP kinase is likely to be responsible for the report of initial burst kinetics by DnaK during ATPase assays (1), similar to our observations, and for the report of an ADP kinase activity by DnaK (2), also similar to our observations. Therefore, the conclusions drawn in these reports, that DnaK hydrolyzes ATP with initial burst kinetics and that DnaK has an ADP kinase or ADP-ATP exchange activity, are erroneous. The NTB hydrolysis activity of NDP kinase is also nucleotide nonspecific (51), 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 (32). The wide range of values for the peptide-, DnaK-, 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.

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 (41). We postulate that because the *ndk::km* disruption only involves an insertion into the *ndk* gene and no deletion within the gene itself some low frequency genetic event occurs randomly 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 s⁻¹ using our assay conditions. This value corresponds well with previously reported *k*₅₀ values for bacterial NDP kinases (51). 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. 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 therefore explains the nonspecific nature with regard to phosphate acceptor substrate that we observed.

NDP kinase is ubiquitous and highly conserved. *E. coli* NDP kinase and human NDP kinase are 43% identical in their amino acid sequences (50). NDP kinases from different organisms are also remarkably similar in their tertiary protein structures (56). It is therefore likely that NDP kinases from different organisms would behave similarly during various chromatographic separations because of 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 (51) 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 (2), 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 mass of 66 kDa (57), 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 (52). 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.

DnaK has a weak physical interaction with His-tagged NDP kinase. The significance of this interaction is not known. DnaK, because it is a molecular chaperone, binds a wide range of substrate peptides (58). The weak interaction with His-tagged NDP kinase could simply result from the nonspecific binding of the chaperone to a second protein. Alternatively, it could be the result of a weak but specific interaction between the two proteins. 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 Brilliant Blue-stained SDS-PAGE gel to co-elute with His-tagged NDP kinase. If the interaction of the proteins in the cytoplasm is specific and physiologically relevant, perhaps NDP kinase modulates the functioning of DnaK as a 16-kDa NDP kinase protein has been suggested to modulate Hac70 (52). An alternative hypothesis is suggested by the observation that NDP kinase and an ATPase with a high level of sequence identity to DnaK are secreted from *Mycobacterium bovis* cells (59). *E. coli* DnaK forms a stable complex with NDP kinase from *M. bovis*, and this interaction could be related to the secretion of these two proteins. The physical interaction between *E. coli* DnaK and NDP kinase, regardless of its significance, may be responsible for the co-purification of small but significant amounts of NDP kinase in DnaK preparations.

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Inferences Concerning the ATPase Properties of DnaK and Other HSP70s Are Affected by the ADP Kinase Activity of Copurifying Nucleoside-diphosphate Kinase

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