Escherichia coli DnaK Protein Possesses a 5'-Nucleotidase Activity That Is Inhibited by ApppppA

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ApppppA and the DnaK protein have both been hypothesized to function in regulating the heat shock response of Escherichia coli. The proposals are that ApppppA serves as a signal (alarmone) to turn on the heat shock response, whereas the DnaK protein is necessary to turn off the heat shock response. A simple model would be that the DnaK protein turns off the response by degrading ApppppA. We disproved this model by demonstrating that the DnaK protein possesses a 5'-nucleotidase activity capable of degrading many cellular nucleotides but not ApppppA. Although ApppppA was not a substrate, it did inhibit the 5'-nucleotidase activity of the DnaK protein. This inhibition may be specific and have biological function since the mutant DnaK756 protein, which is defective in turning off the heat shock response, is partially desensitized to ApppppA inhibition. These findings led us to consider other possible mechanisms for ApppppA and the DnaK protein in heat shock regulation.

ApppppA is an unusual nucleotide present at very low levels in exponentially growing procaryotic and eucaryotic cells (3, 16). The level of ApppppA and other adenylylated nucleotides rises rapidly and dramatically when cells are exposed to an oxidation stress (3, 13). Because of this, it has been proposed that ApppppA may function as an alarmone to activate cellular defense mechanisms against oxidation damage. Interestingly, ApppppA levels also rise in cells exposed to high temperature or to ethanol. Both of these treatments lead to an induction of the heat shock response (14).

Besides elevated synthesis of ApppppA, other observations also indicate a close relationship between oxidation stress and heat shock. Several examples demonstrating cross adaptation to sublethal doses of heat and oxidizing agents have been published (4, 14). More recently, the proteins induced in response to these stresses in Salmonella typhimurium have been determined by two-dimensional gel analysis. Some of the proteins induced are specific to heat or oxidation stress, but other proteins are common to both stress responses. The DnaK protein analog of S. typhimurium (D64a) is a dominant protein produced under both stresses (4).

The DnaK protein from Escherichia coli is the most studied and perhaps the most interesting of the so-called heat shock proteins. It has been purified and characterized biochemically and found to have four assayable activities. (i) It is essential for an in vitro λ replication system dependent on the bacteriophage-coded O and P proteins (17, 18). (ii) It is active in phage M13 DNA replication in vitro in a reaction system also dependent on the λ O and P proteins (12). (iii) It autophosphorylates at a threonine residue (18). (iv) It has a weak DNA-independent ATPase activity (17, 18). DNA sequence analysis indicates that the DnaK protein is 48% identical to the Hsp70 protein of Drosophila melanogaster (1). DNA hybridization and immunological cross-reactivity studies indicate that the Hsp70 protein and its gene (or in some cases, gene families) are present throughout the biological kingdoms: from E. coli to Saccharomyces cerevisiae, Dictyostelium discoideum, D. melanogaster, chickens, hu-

mans, and even the archaebacterium Methanosarcina barkeri (1, 11). The function of the Hsp70 protein is not known but in two organisms studied, E. coli (15) and D. melanogaster (6), it appears to play an essential role in turning off the heat shock response.

We investigated the possibility of a mechanistic connection between the proposed antagonistic roles of ApppppA in turning on the heat shock response and of the DnaK protein in turning off the heat shock response. Because the ATPase activity of the DnaK protein is very weak, we suspected that its preferred in vivo substrate might be ApppppA (adenylylated ATP). Therefore, we asked whether the DnaK protein might modulate the response by enzymatically hydrolyzing ApppppA. Our study was initiated to test this hypothesis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. E. coli K-12 strain 1A2, a prototrophic isolate derived from strain W3110, was used in these studies. Methods of cell culture and labeling were as previously described (2), except that the culture medium was composed of 100 mM triethanolamine (pH adjusted to 7.2 with HCl), 25 mM glucose, 10 mM NH_4Cl, 10 mM KCl, 200 μm NaH_2PO_4, 50 μM Na_2SO_4, 50 μM $MgCl_2$, and 20 μM each of the standard 19 amino acids (no cysteine). Adenylylated nucleotide synthesis was induced by adding 1 mM $CdCl_2$ (3) to the culture.

Purification of proteins. The purification of the DnaK and DnaK756 proteins from the isogenic bacterial strains B178 dnaK and B178 dnaK756 has been previously described (17). Both protein preparations were greater than 85% pure as judged by Coomassie blue staining.

Enzymatic digestions and nucleotide analysis. Extracts were prepared from ^{32}P-labeled cells as previously described, by using formic acid neutralized with N-ethylmorpholine (2). Digestions of nucleotide extracts were done in Eppendorf tubes containing 10 μl of extract, 0.5 μl of 100 mM $MgCl_2$, 0.5 μl of 100 mM β-mercaptoethanol, and 0.5 μl of purified DnaK protein (200 ng/μl). The digestions were carried out overnight at different temperatures. After incubation, the digestion mixture was resolved on

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polyethyleneimine cellulose thin-layer chromatography (TLC) sheets, by using solvent Ga followed by solvent Sb (2). The TLC sheets were then used to expose X-ray films (Kodak XRP) by autoradiography.

Digestions of ^{14}C-labeled nucleotides were done in 5-μl volumes in Eppendorf tubes containing 100 mM Tris hydrochloride buffer, 5 mM MgCl$_2$, 5 mM β-mercaptoethanol, 340 ng of purified DnaK or DnaK756 protein, and 12 nCi of ^{14}C-nucleotide. The digestions were carried out overnight at different temperatures. After incubation, the digestion products were resolved on polyethyleneimine cellulose TLC sheets, by using a solvent of 0.5 M Tris–0.3 M HCl. X-ray films were exposed by autoradiography. [8-^{14}C]ATP, [8-^{14}C]ADP, and [8-^{14}C]AMP (50 mCi/mmol) were purchased from ICN Pharmaceuticals, Inc. [U-^{14}C]GMP (500 mCi/mmol) was from Amersham Corp.

RESULTS

Nucleotidase activity of the DnaK protein. In a previous study (18), ATP and dATP were found to be poor substrates for hydrolysis [to (d)ADP plus P$_i$] by the DnaK protein; however, the specificity of the enzymatic activity was not broadly surveyed. One of the proposed uses for the nucleotide analysis method of Bochner and Ames (2) is to facilitate studies of the specificity of proteins with nucleotidase activity. To use this method, an extract is prepared from ^{32}P-labeled *E. coli* cells which contains all the nucleotides normally present in exponentially growing cells, with the relative proportions representative of the in vivo pools. Two aliquots of this extract are compared. One aliquot is incubated without protein addition as a control. The second aliquot is incubated with a purified protein, in this case the DnaK protein. After incubation, the nucleotides remaining are resolved and analyzed by TLC and autoradiography. If a spot on the autoradiogram decreases in intensity or disappears completely, this indicates that its corresponding nucleotide is a substrate for the enzyme.

Using this technique, we found that the DnaK protein is not a specific ATPase but a rather nonspecific 5'-nucleotidase. It is clearly active against 5'-triphosphates (ATP, dATP, GTP, dGTP, UTP), 5'-monophosphates (AMP, GMP, CMP, UMP), and UDP (Fig. 1A and B). The 5'-diphosphates (except UDP) do not disappear from the autoradiogram because they are formed from triphosphate hydrolysis faster than they hydrolyzed. ADP can be degraded by the DnaK protein (Fig. 2), but in general the protein prefers tri- and monophosphate nucleotides over diphosphates. Except for the uridine nucleotides and CMP, most pyrimidine nucleotides (CTP, dCTP, dTTP, CDP, dCDP, dTDP) are relatively resistant to hydrolysis. The enzyme specifically hydrolyzes 5'-phosphates and does not hydrolyze external phosphates in 2' (e.g., NADPH) or 3' (e.g., acetyl coenzyme A) linkages. The enzyme also fails to hydrolyze internal 5'-linked phosphate esters (e.g., NAD).

Several proteins which may complex with and modify the function of the DnaK protein in vivo (7) were also tested in this assay system but had no detectable effects. Purified fractions containing the *E. coli* heat shock proteins DnaJ, GroEL, and GroES were added to the ^{32}P-nucleotide extract either individually, paired with DnaK, or in combinations along with DnaK. None of the proteins exhibited nucleotidase activity, and none of the proteins or protein combinations altered the nucleotidase activity of the DnaK protein (data not shown).

The DnaK protein is not an AppppA hydrolase. To test whether AppppA is a preferred substrate for the DnaK protein, the same procedure was repeated with an extract prepared from cells stressed so that they had elevated levels of AppppA and other adenylylated nucleotides. The results (Fig. 1C and D) demonstrate that none of the adenylylated nucleotides is detectably hydrolyzed by the DnaK protein. Thus, our hypothesis is that the DnaK protein turns off the heat shock response by degrading AppppA is incorrect.

Nucleotidase activity of the DnaK protein is inhibited by AppppA. Although AppppA is not a substrate for the DnaK nucleotidase, it does inhibit its activity. Data demonstrating this are shown in Fig. 1E and F. A set of digestions with the DnaK protein was performed in which various adenosine nucleosides (adenosine, AMP, ADP, ATP, and AppppA) were added to the digestion mixture at a concentration of 170 μM (a level typical of AppppA pools in stressed cells [3]) and compared with a control with no addition. The control (Fig. 1D) and the digestion with added adenosine (Fig. 1E) were identical, with normal digestion of nucleotides. The digestions with added AMP, ADP, and ATP were completely inhibited (data not shown), but this was to be expected since they are all substrates for the enzyme and the excess unlabeled nucleotide out-competes the ^{32}P-labeled nucleotides as substrates. Surprisingly, the digestion of the nucleotides in the reaction with added AppppA was also completely inhibited (Fig. 1F) even though AppppA is not a substrate for the hydrolyzing activity of the DnaK protein.

Although these results indicated that AppppA is an inhibitor of the DnaK nucleotidase activity in vitro, there was no direct evidence indicating that this inhibition was specific to AppppA and potentially of biological relevance.

5'-Nucleotidase activity of the DnaK756 protein is partially desensitized to AppppA inhibition. If, in fact, the inhibition of the DnaK 5'-nucleotidase by AppppA is involved in the mechanism of regulating the heat shock response, one might expect to see an altered interaction with the DnaK756 protein. This mutant DnaK protein functions improperly and is defective in turning off the heat shock response (15). We therefore asked whether the sensitivity of the mutant DnaK protein to AppppA also was altered.

First, experiments were performed to compare the nucleotidase activity of the DnaK756 protein with that of the DnaK protein. Two-dimensional nucleotide analysis indicated that the DnaK756 protein was relatively less active against ATP and more active against ADP (data not shown). This altered specificity is demonstrated more clearly in the experiment shown in Fig. 2. The activities of the DnaK and the DnaK756 protein were compared at 32°C against single ^{14}C-labeled nucleotide substrates. The results indicate that the DnaK protein is relatively more active than the DnaK756 protein against ATP, whereas it is relatively less active against ADP and AMP. Both proteins are active against GMP. Other incubation temperatures (23 and 43°C) were also examined since the DnaK756 mutation results in a temperature-sensitive growth defect at 43°C (8). However, although the activities of both proteins increased with increasing temperatures, no alterations in specificity were observed with varying temperatures (data not shown).

To test whether the inhibition of nucleotidase activity by AppppA was altered in the case of the DnaK756 protein, an experiment was performed with [^{14}C]GMP as a substrate, since it was actively hydrolyzed by both the wild-type and the mutant proteins. At pH 7.4, the DnaK756 protein appears to be slightly desensitized to AppppA inhibition compared with the DnaK protein (Fig. 3, lane c versus lane e). However, at pH 8.4 a relative insensitivity of the DnaK756 protein is clearly observable (lane h versus lane j).
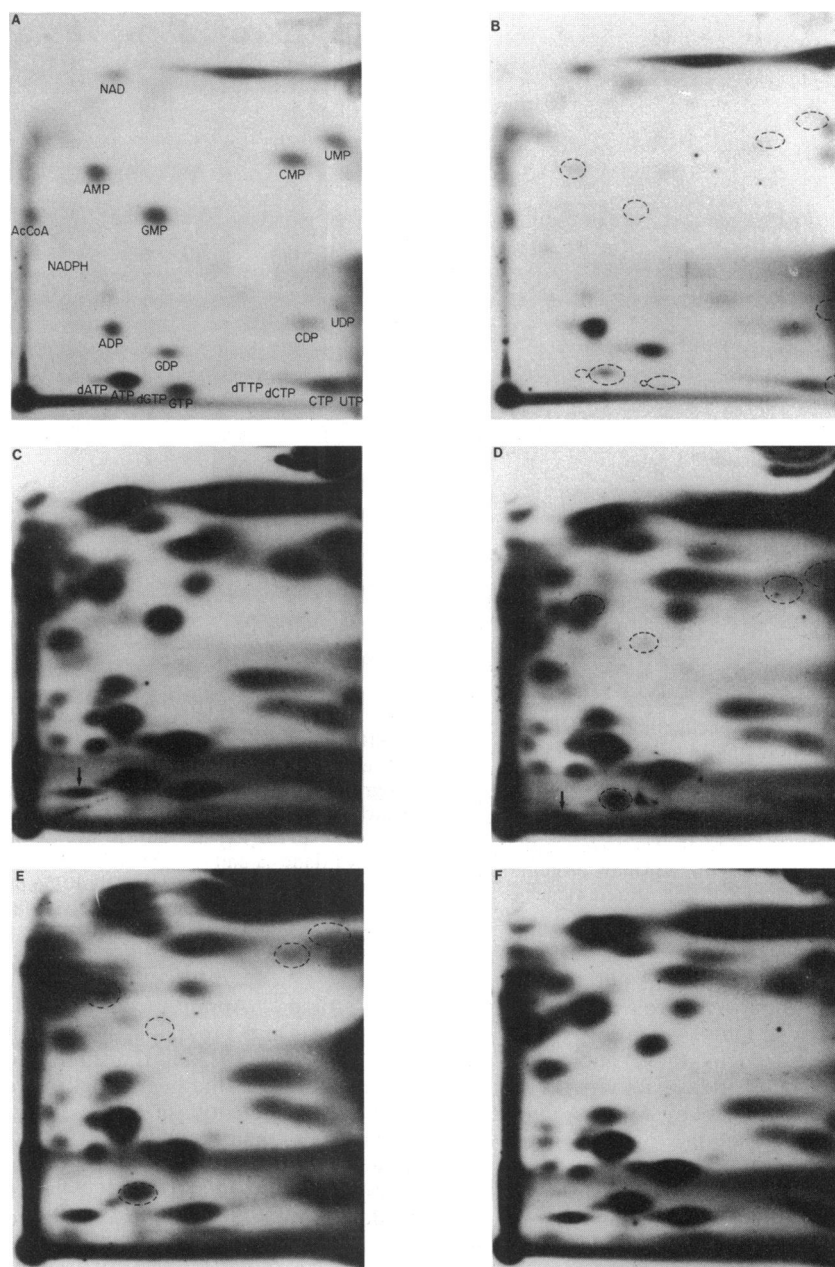
FIG. 1. Nucleotidase activity of the DnaK protein and its inhibition by AppppA. Shown are autoradiograms exposed from two-dimensional TLCs as described in Materials and Methods. An *E. coli* extract from exponential phase cells was incubated at 23°C with (A) no enzyme, or (B) DnaK protein. An extract from CdCl$_2$-inhibited *E. coli* was incubated at 23°C with (C) no enzyme, (D) DnaK protein, (E) DnaK protein and 170 μM adenosine, or (F) DnaK protein and 170 μM AppppA. The dashed circles highlight the spots that have been degraded. Five adenylylated nucleotides are present as spots in the lower left corner of panels C, D, E, and F. AppppA is indicated by the arrow in panels C and D. AcCoA, Acetyl coenzyme A.

DISCUSSION

It is clear from our results that the DnaK protein is not an AppppA hydrolase and that the simple model of the DnaK protein turning off the heat shock response by degrading AppppA is incorrect. However, our results also indicate that AppppA, at least in vitro, does affect an assayable activity of the DnaK protein. Furthermore, some evidence supports the idea that this effect may be specific, indicating a possible in vivo function. The principal findings can be summarized as follows. (i) The catalytic activity of the DnaK protein is not a specific ATPase but instead a nonspecific 5'-nucleotidase with a preference for tri- and monophosphates and for purines over pyrimidines (especially cytidine). (ii) The in vitro nucleotidase activity of the DnaK protein is inhibited by a concentration of AppppA (170 μM) seen in vivo in stressed cells (3). (iii) The mutant DnaK756 protein is relatively more active against di- and monophosphates and less active against triphosphates than is the DnaK protein. (iv) The DnaK756 protein is relatively desensitized, espe-
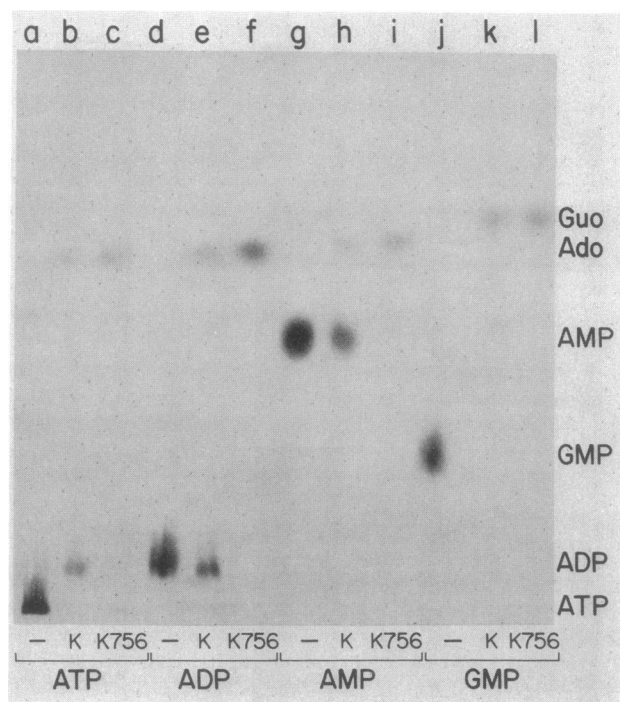
FIG. 2. Comparative nucleotidase specificities of the DnaK protein and the DnaK756 protein. Shown is an autoradiogram exposed from a one-dimensional TLC as described in Materials and Methods. Digestions were carried out at pH 8.0 at 32°C. The ^{14}C-nucleotide substrates were ATP (lanes a to c), ADP (lanes d to f), AMP (lanes g to i), and GMP (lanes j to l). They were either not digested (lanes a, d, g, and j), digested with the DnaK protein (lanes b, e, h, and k), or digested with the DnaK756 protein (lanes c, f, i, and l). Guo, Guanosine; Ado, adenosine.
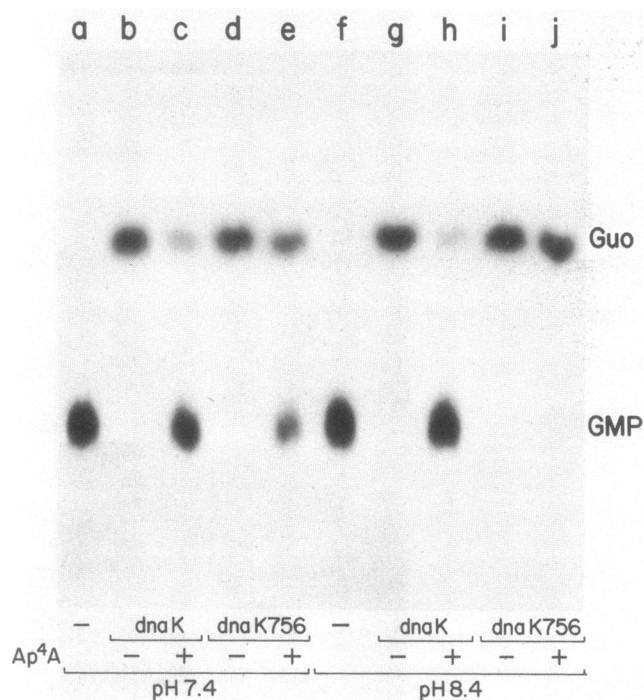

FIG. 3. Comparison of the DnaK protein and the DnaK756 protein in sensitivity to inhibition by ApppppA. Shown is an autoradiogram exposed from a one-dimensional TLC as described in Materials and Methods. Digestions were carried out at pH 7.4 (lanes a to e) or pH 8.4 (lanes f to j) at 23°C. [^{14}C]GMP was either not digested (lanes a and f), digested with the DnaK protein (lanes b, c, g, and h), or digested with the DnaK756 protein (lanes d, e, i, and j) without (lanes a, b, d, f, g, and i) or with (lanes c, e, h, and j) 170 μM ApppppA added. Ap^{4}A, ApppppA.

cially at pH 8.4, to inhibition by ApppppA. A different protein which may be the in vivo ApppppA hydrolase has recently been purified and characterized (10).

Two new questions are raised by these findings. What function, if any, does the 5'-nucleotidase activity serve, and how might inhibition of DnaK activity by ApppppA modulate the heat shock response? A potential role for the newly recognized 5'-nucleotidase activity may be found in the assembly reaction of a pre-primosome on λ DNA and on M13 single-stranded DNA. The pre-primosome assembly, directed by the λ phage-coded O and P proteins and the E. coli DnaB, DnaK, and DnaJ proteins, requires a source of energy, satisfied by ribonucleotide triphosphates (12). Since two of the proteins, DnaB and DnaK, are capable of hydrolyzing triphosphates, it is not clear whether one or both are required in this assembly system. In view of the nonspecificity of the 5'-nucleotidase activity of DnaK protein, it will be interesting to see whether di- or monophosphate nucleotides can substitute as energy sources in this assembly reaction.

Bacterial dnaK mutants overproduce heat shock proteins at low temperature and fail to turn off the heat shock response at high temperatures (15; unpublished results). These observations have led to the proposal that the DnaK protein is an antagonist of the HtpR protein, the σ^{32} factor of the RNA polymerase (9) which enables the enzyme to transcribe heat shock promoters (5). Because the DnaK protein is capable of autophosphorylation (18), an interesting possibility is that it modulates the heat shock response in vivo by phosphorylating one or more E. coli proteins, such

as σ^{32} or σ^{70}. ApppppA could therefore play a role in the ability of DnaK to phosphorylate itself or other E. coli regulatory proteins, and this interaction may be important in the regulation of the heat shock response. The observation that the 5'-nucleotidase of the DnaK756 mutant protein is partially desensitized to inhibition by ApppppA may also be reflected in the ability of ApppppA to modulate the phosphorylation activity of the DnaK756 protein. Experiments are under way to test these possibilities both in vivo and in vitro.

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ADDENDUM IN PROOF

A recent publication (R. M. Pinto, J. Canales, N. A. G. Sillero, and A. Sillero, Biochem. Biophys. Res. Commun. 138:261–267) reports that a cytosylic 5'-nucleotidase from Artemia embryos is activated by micromolar levels of ApppppA.

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