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The dnaK protein of *Escherichia coli* possesses an ATPase and autophosphorylating activity and is essential in an *in vitro* DNA replication system

(O protein/P protein/kinase activity/single-stranded DNA replication)

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ABSTRACT **The *Escherichia coli dnaK* gene product, originally defined by mutations that blocked λ phage DNA replication, is known to be necessary for *E. coli* viability. We have purified dnaK protein to homogeneity and have demonstrated that it possesses a weak DNA-independent ATPase activity, which results in the production of ADP and P_i. The proof that this ATPase activity is encoded by the *dnaK*$^+$ gene relies primarily on the fact that the *dnaK756* mutation results in the production of an ATPase activity with altered physical properties. The dnaK protein is phosphorylated *in vitro* and *in vivo*, probably as a result of an autophosphorylation reaction. The λ O and P replication proteins were shown to interact *in vitro* with the dnaK protein. The ATPase activity of the dnaK protein was inhibited by purified λ P protein and stimulated by purified λ O protein. Moreover, the dnaK protein participates in the initiation of DNA synthesis in an *in vitro* DNA replication system that is dependent on the O and P proteins. Anti-dnaK protein immunoglobulin specifically inhibited DNA synthesis in this system.**

The study of host–virus interactions has been greatly facilitated through the isolation of host mutants that block viral growth. Through the use of indirect or direct selections, various investigators have uncovered five *Escherichia coli* genes whose products are necessary for bacteriophage λ DNA replication: *dnaB*, *dnaK*, *dnaJ*, *grpD*, and *grpE* (1–4). Of these genes, only *dnaB* has been convincingly shown to be essential for *E. coli* DNA replication (5). Both the *dnaK*$^+$ and *dnaJ*$^+$ gene products are essential for bacterial growth as well, because some mutations in these genes block colony formation at 42°C. Both DNA and RNA bacterial metabolism are affected in *dnaK*$^-$ (4, 6) and *dnaJ*$^-$ mutants (7, 8). The effect on RNA metabolism is unique and not shared by mutations in the rest of the *dna* genes of *E. coli* (5). Mutations in λ phage that enable it to grow on *dnaK*$^-$ hosts map in the *P* gene, which suggests an interaction between the host dnaK protein and the phage P protein (1, 2). The *dnaK* gene has been cloned into λ phage (2), and its gene product has been identified by $NaDodSO_4$/polyacrylamide gel electrophoresis (9) and shown to be identical to protein B66.0 (10), one of the heat shock proteins of *E. coli* (11–13). In this communication, we report that the dnaK$^+$ protein (*i*) possesses a weak DNA-independent ATPase activity that is modulated *in vitro* by the λ O and P proteins; (*ii*) is phosphorylated both *in vitro* and *in vivo*, probably as a result of an autophosphorylation reaction; and (*iii*) is active in an *in vitro* replication system (unpublished results) that is dependent on the λ O and P proteins.

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METHODS AND MATERIALS

Strains. The bacteria, bacteriophage, and plasmid strains used in this work have been described (2, 9, 10, 14, 15).

Purification of dnaK$^+$ Protein. The details of the purification will be published elsewhere. Briefly, 12 g of wet paste of B178(pMOB45*dnaK*$^+$) bacteria was lysed according to Shlomai and Kornberg (16). The supernatant was passed through a DE52 column equilibrated with 0.1 M ammonium sulfate/50 mM Tris·HCl, pH 7.8/0.1 mM EDTA/10% glycerol (vol/vol) to remove excess DNA. The void volume was treated with 47% saturated ammonium sulfate, and the resulting precipitate was suspended and passed through a phosphocellulose column. The void volume was added to a DEAE-Sephacel column, which was eluted with a gradient of 50 mM–0.4 M NaCl. Fractions containing the dnaK protein were pooled and passed through a hydroxylapatite column. The dnaK protein was eluted with 50 mM phosphate buffer (pH 7.5), concentrated, and then centrifuged through a 20–40% linear glycerol gradient for 26 hr at 250,000 × *g*.

ATPase Assay. Purified dnaK protein was assayed essentially as described by Shlomai and Kornberg (16). The 25-μl reaction mixture contained 50 mM Tris·HCl, pH 8.0/2 mM $MgCl_2$/bovine serum albumin (200 μg/ml)/5 mM 2-mercaptoethanol/5% glycerol (vol/vol)/0.1 mM unlabeled ATP/0.05 μCi of [α-^{32}P]-ATP (1 Ci = 3.7×10^{10} Bq; Amersham). Reactions were carried out for 20 min at 30°C. The ATPase activity of crude extracts was determined after passage through the DE52 column and precipitation with ammonium sulfate. The reaction buffer was the same as for the purified protein except that 10 mM *N*-ethylmaleimide, rifampicin at 50 μg/ml, and nalidixic acid at 100 μg/ml were added and 2-mercaptoethanol was omitted.

***In Vitro* Phosphorylation of dnaK Protein.** Approximately 5 μg of purified dnaK protein was incubated for 30 min at 37°C in a 50-μl reaction mixture consisting of 50 mM Mes buffer, pH 6.2/5 mM 2-mercaptoethanol/5 mM $MgCl_2$/10% glycerol/1 μM unlabeled ATP/10 μCi of [γ-^{32}P]ATP. The reaction was stopped by the addition of 200 μl of 20% trichloroacetic acid and 1 μl of 10% deoxycholate. After 30 min at 0°C, the sample was centrifuged for 10 min at 15,000 × *g*. The pellet was washed once with 1 ml of acetone at −20°C, dried under vacuum, suspended in 50 μl of $NaDodSO_4$ sample buffer, boiled for 5 min, and run on a 12.5% $NaDodSO_4$/polyacrylamide gel.

Phosphoamino Acid Analysis. *In vitro* labeled dnaK protein (5 μg) was precipitated, washed, and hydrolyzed as described by Maness and Levy (17). The hydrolysate was mixed with

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phosphoserine, phosphothreonine, and phosphotyrosine standards, applied to Whatman 3MM paper, and electrophoresed in a buffer (pH 3.5) containing 5% acetic acid and 0.5% pyridine as described by Clinton and Huang (18).

Electrophoresis. One- and two-dimensional gel electrophoresis were carried out as described (10). For native isoelectric focusing of dnaK protein, the sample was run in the absence of urea, with 0.5% Ampholines (pH 4–6) (LKB). Glycerol (20%) was included to stabilize the proteins.

RESULTS

ATPase Activity of the dnaK Protein. The 5.3-kilobase *Hin*dIII DNA fragment carrying the intact *dnaK*$^+$ gene along with its promoter was cloned from the λ *dnaK*$^+$ transducing phage (2) onto the "runaway" plasmid pMOB45 (15). Bacteria carrying this plasmid contained 50-fold amplified levels of the dnaK protein (M_r = 77,000). By using such bacterial strains as starting material, we purified the dnaK protein to homogeneity. The purified protein was shown to migrate in two-dimensional gels with authentic dnaK protein labeled with [^{35}S]methionine after infection of UV-irradiated bacteria with λ*dnaK*$^+$ transducing phage (9). During purification, fractions were monitored for the M_r 77,000 protein on NaDodSO$_4$/polyacrylamide gels because no enzymatic activity of dnaK protein was known. The purified protein was found to hydrolyze ATP to ADP and P_i. The pH optimum of the reaction was 8.8 (Fig. 1). The hydrolysis of ATP was linear for up to 30 min at 30°C. The apparent K_m for ATP was about 0.2 mM. The ATPase activity was weak inasmuch as only 15–20 nmol of ATP were hydrolyzed per mg of protein per min at 30°C. This represents a turnover of only 1 ATP molecule per min per active site. dATP was found to be a poor substrate; its rate of hydrolysis was one-fifth that of ATP. The ATPase activity could not be separated from the purified dnaK protein under a variety of experimental conditions.

Tests of the effects of a variety of compounds on the ATPase activity showed the following. (*i*) Native or denatured DNA did not stimulate the ATPase activity. This result was somewhat unexpected because other DNA replication proteins, such as the dnaB protein, protein n′, helicase 1, and helicase 2, exhibit an ATPase activity that is stimulated by DNA (5). (*ii*) Approximately 10% of the activity remained after a 10-min incubation of the dnaK protein at 95°C. Using this characteristic property as a criterion, we repurified dnaK protein and assayed for heat-resistant ATPase activity. The heat-resistant ATPase and the dnaK protein copurified. (*iii*) The ATPase activity was resistant to *N*-ethylmaleimide, which suggests that the dnaK protein does not require free sulfhydryl groups for enzymatic activity. (*iv*) The ATPase activity of dnaK protein was retained in immunocomplexes with anti-dnaK protein antibodies.

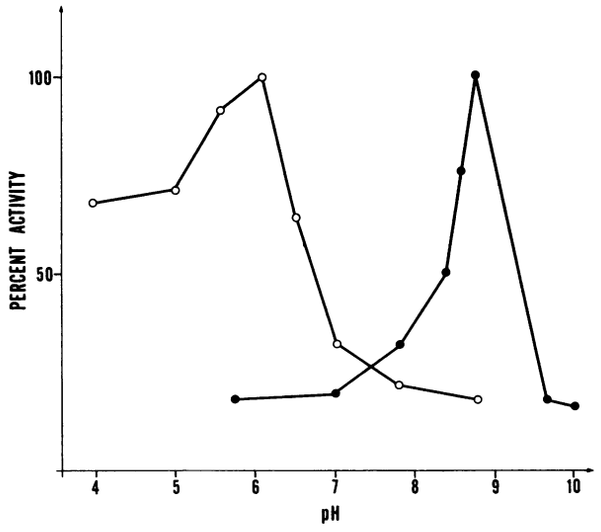

FIG. 1. The dnaK protein possesses both ATPase and kinase activities. ●, pH optimum of the ATPase activity; ○, pH optimum of the autophosphorylating activity of purified dnaK protein.

The evidence that the ATPase activity resides in the dnaK protein and not another contaminating polypeptide rests on the following observations. (*i*) The levels of ATPase activity in crude extracts from a dnaK756 mutant, after immunoprecipitation with an anti-dnaK protein antibody preparation, were 10–20% of those found in lysates of dnaK$^+$ strains (data not shown). (*ii*) Bacterial strains carrying the pMOB45dnaK$^+$ plasmid overproduce both dnaK protein and dnaK-specific ATPase activity to the same extent. Isogeneic bacteria carrying pMOB45 alone did not show this increase (data not shown). (*iii*) The ATPase activities of dnaK$^+$ and dnaK756 proteins can be separated on a nondenaturing isoelectric focusing gel (Fig. 2). The positions of the ATPase activities were identical to the positions of the corresponding wild-type or mutant proteins (10). Fig. 2*C* shows that mixed extracts from dnaK$^+$ and dnaK756 strains show two peaks of ATPase activity, as expected.

***In Vitro* Interactions with Purified Replication Proteins of λ Phage.** Previous genetic studies have suggested that the *E. coli* dnaK protein interacts with the λ P replication protein, because mutations in λ phage that enable it to propagate on dnaK756 bacteria map in the *P* gene (1, 2). The only two phage gene products known to be essential for λ DNA replication are the

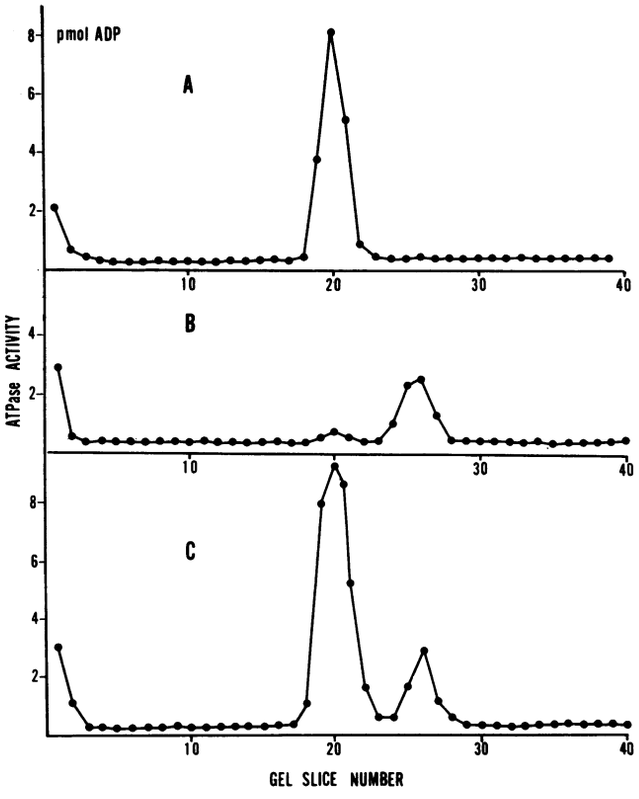

FIG. 2. The heat-resistant ATPase activity is encoded by the *dnaK* gene. The ATPase activity of dnaK$^+$ and dnaK756 crude extracts after separation on nondenaturing isoelectrofocusing gels. (*A*) dnaK$^+$ extracts. (*B*) dnaK756 extracts. (*C*) A mixture of dnaK$^+$ and dnaK756 extracts. The gel was divided into 40 slices. Each slice was suspended in ATPase buffer (25% glycerol minus the ATP), incubated at 95°C for 3 min, and assayed for ATPase activity.

O and P proteins (19). Because of the availability of these proteins in a homogeneous form, we examined their effects on the ATPase activity of the purified dnaK protein. Purified P protein inhibited the ATPase activity of the dnaK protein (Fig. 3) with maximal inhibition being achieved at a 1:1 molar ratio. Prior treatment of P protein with *N*-ethylmaleimide did not affect its capacity to inhibit the dnaK protein ATPase activity. This result differs from that obtained previously for P protein inhibition of the ATPase activity of dnaB protein (20). In contrast to P protein, the purified O protein stimulated the ATPase activity of dnaK protein (Fig. 4). These results suggest that both of the λ phage replication proteins interact with the host dnaK protein.

***In Vitro* Phosphorylation of dnaK Protein.** When purified preparations of dnaK proteins were incubated *in vitro* with [γ-^{32}P]ATP, a portion of the radioactivity was found to be tightly associated with the protein. This association was resistant to boiling in 3% NaDodSO$_4$/5% 2-mercaptoethanol (Fig. 5). Neither [α-^{32}P]ATP nor [^{14}C]ATP labeled the protein, which suggests that the dnaK protein was phosphorylated by ATP. The labeling of the dnaK protein varied with the pH of the reaction (Fig. 1). The phosphorylation reaction, in contrast to the ATPase reaction, was optimal at pH 6.2. At this pH, more than 10% of the dnaK protein was phosphorylated after incubation for 30 min at 37°C. To identify the phosphorylated residue, dnaK protein was labeled with [γ-^{32}P]ATP and hydrolyzed. The label migrated with the phosphothreonine marker (Fig. 6), demonstrating that the dnaK protein was phosphorylated at a threonine residue.

The phosphorylation of the dnaK protein required Mg^{2+}, was not inhibited to a large extent by prior heating for 10 min at 95°C, and was still observed in immunocomplexes with anti-dnaK protein immunoglobulin. The latter result strongly suggests, but does not prove, that the phosphorylation of dnaK protein reflects an endogenous autophosphorylating activity rather than the action of a contaminating kinase. The mutant dnaK756 protein was also capable of being autophosphorylated after precipitation with anti-dnaK protein antibodies. The phosphorylation reaction was highly specific because added casein, bovine serum albumin, lysozyme, or IgG molecules were

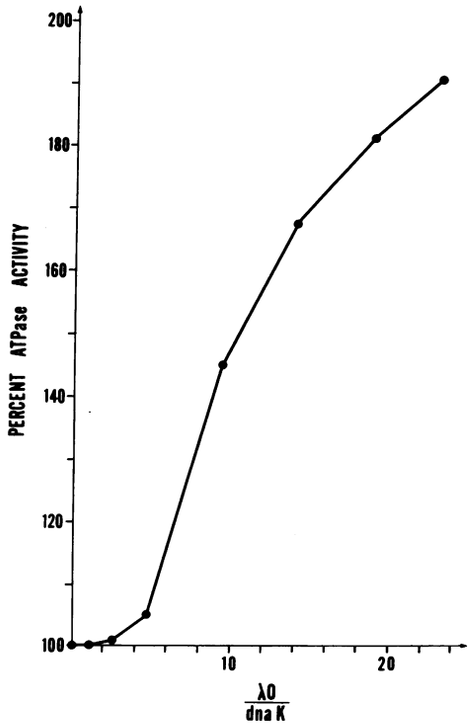

FIG. 4. *In vitro* effects of purified λ O protein on the ATPase activity of purified dnaK protein. The percentage of ATPase activity is shown as a function of the O/dnaK polypeptide molar ratio.

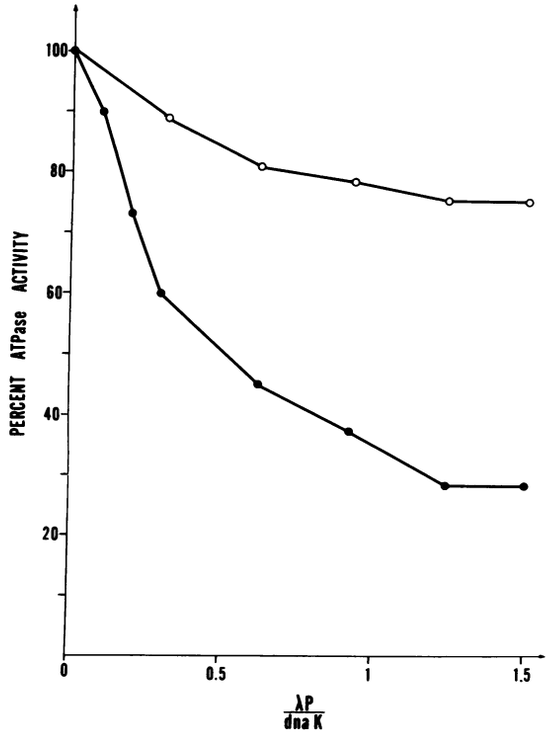

FIG. 3. *In vitro* effects of purified λ P protein on the ATPase activity of purified dnaK protein. ●, Incubation of 1 μg of dnaK protein with increasing amounts of P protein. ○, The purified P protein was inactivated prior to incubation by heating at 65°C for 5 min. The percentage of ATPase activity is shown as a function of the P/dnaK polypeptide molar ratio.

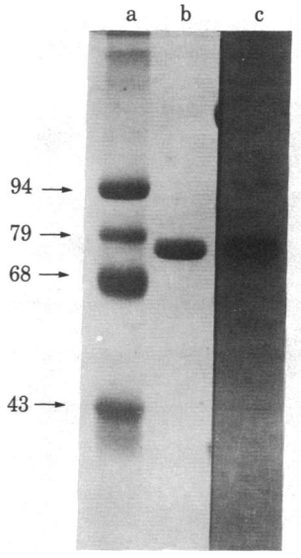

FIG. 5. *In vitro* phosphorylation of dnaK protein. Purified dnaK protein was phosphorylated *in vitro* after incubation with [γ-^{32}P]ATP. Lanes: a, protein size standards ($M_r \times 10^{-3}$); b, Coomassie blue staining of ^{32}P-labeled dnaK protein after electrophoresis in 10% NaDodSO$_4$/polyacrylamide gel; c, autoradiogram of the protein in lane b. The positions of stained dnaK protein and radioactivity coincide.

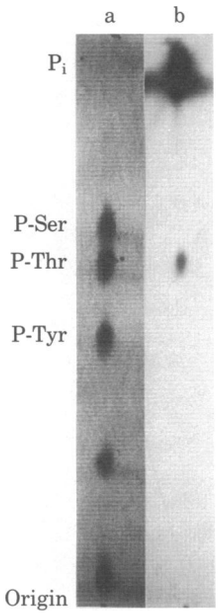

FIG. 6. Purified dnaK protein is phosphorylated *in vitro* at a threonine residue. [γ-^{32}P]ATP-labeled dnaK protein was analyzed for phosphoamino acid content. The positions of the ninhydrin-stained phosphothreonine (lane a) and radioactivity (lane b) are identical.

not phosphorylated to an appreciable extent by the dnaK protein. Phosphorylation of both the wild-type and dnaK756 proteins was also demonstrated *in vivo* after labeling bacteria with $^{32}P_i$ (21, 22) (Fig. 7). This phosphorylation resulted in an acidic shift in the isoelectric point of the protein. The magnitude of the shift was shown to be identical to that observed with *in vitro* ^{32}P-labeled dnaK protein. Less than 5% of the total dnaK protein was present in the phosphorylated form *in vivo*, as judged by the intensity of protein staining.

dnaK Protein Is Active in an *in Vitro* DNA Replication System. We have recently discovered that the purified λ O and P replication proteins, in conjunction with several host replication proteins, promote the rifampicin-resistant initiation of DNA synthesis on single-stranded DNA templates (unpublished results). An antibody preparation directed against the dnaK protein was tested for its capacity to inhibit the λ O and P protein-dependent conversion of M13 mp7 single-stranded DNA to the duplex replicative form. Anti-dnaK protein immunoglobulin was incubated at 0°C for 15 min with 200 μg of an *E. coli* protein fraction (fraction 2; ref. 23). The capacity of the antibody-treated *E. coli* fraction to support the replication of M13 mp7 DNA was measured in the presence and absence of rifampicin. The DNA synthesis observed in the absence of rifampicin was not inhibited by incubation with the anti-dnaK protein antibody preparation (Fig. 8). This DNA synthesis represents M13 replication via the physiological RNA polymerase-dependent pathway (24), which is known to be independent of the *E. coli* dnaK protein. In contrast, the anti-dnaK protein IgG blocked the λ O- and P-protein-dependent DNA synthesis almost completely. This rifampicin-resistant DNA synthesis could be restored, however, by subsequent addition of excess dnaK protein (Fig. 8), proving that the purified dnaK protein is essential for this novel initiation activity. Prior incubation of the dnaK protein

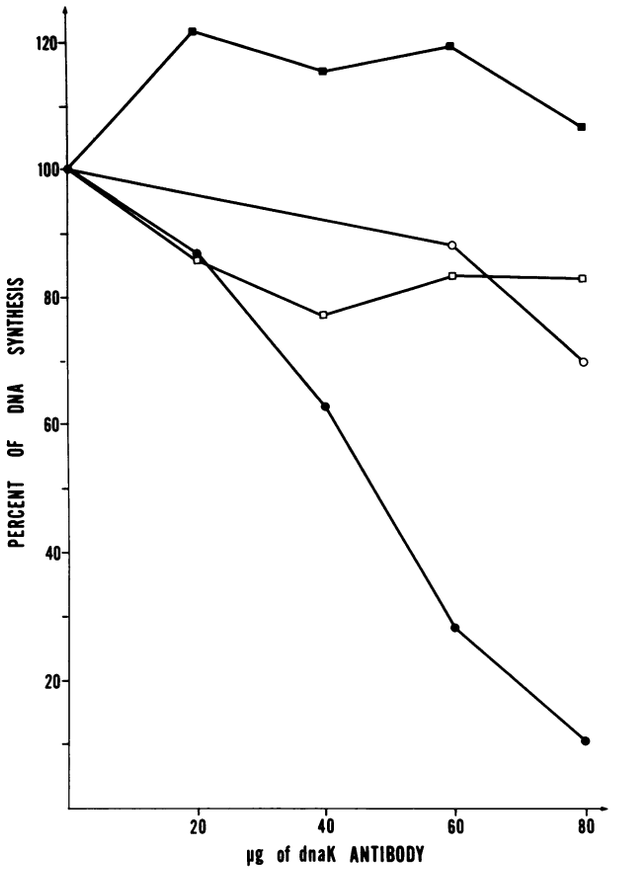

FIG. 8. dnaK protein is active in an *in vitro* DNA replication system. The reaction conditions of the *in vitro* conversion of single-stranded M13 mp7 DNA (500 pmol of nucleotide equivalents per reaction mixture) to a double-stranded form were virtually identical to those described for the *in vitro* replication of λdv (14). Purified O (300 ng) and P (40 ng) proteins were added to all reaction mixtures prior to initiation of DNA synthesis. DNA synthesis was monitored by the incorporation of [^{3}H]dTTP to an acid-insoluble form in the presence of various amounts of anti-dnaK protein IgG-purified rabbit antibodies. ■, DNA synthesis in the absence of rifampicin; ●, DNA synthesis in the presence of rifampicin at 20 μg/ml; ○, DNA synthesis in the presence of rifampicin and 2 μg of exogenous purified dnaK protein; □, DNA synthesis in the presence of rifampicin and nonimmune IgG-purified antibodies. One hundred percent of the M13 reaction without rifampicin represents 227 pmol of dNMP incorporated into trichloroacetic acid-insoluble material. One hundred percent of the O and P protein-dependent system (in the presence of rifampicin) represents 130 pmol of incorporated dNMP.

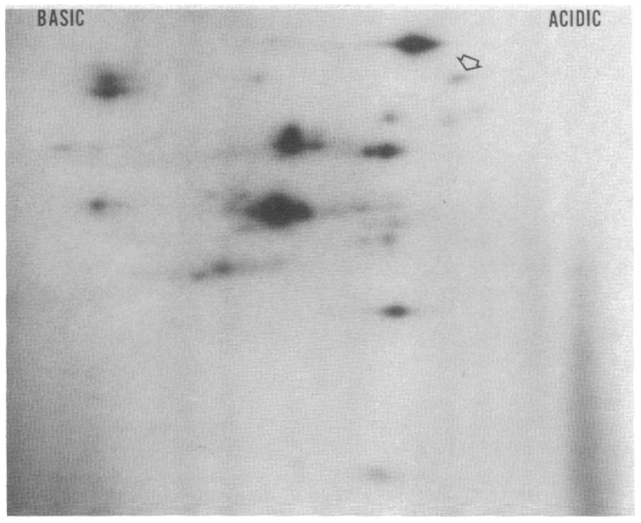

FIG. 7. *In vivo* phosphorylation of dnaK protein. Two-dimensional electrophoresis of $^{32}P_i$-labeled dnaK$^+$ bacteria. The position of phosphorylated dnaK protein is identified with an open arrow.

for 5 min at 92°C blocked its capacity to restore DNA synthesis in the above system.

DISCUSSION

A biological role for the weak ATPase activity associated with the dnaK protein has not yet been established. It could be that the ATPase activity is enhanced *in vivo* through an interaction(s) with other intracellular components. The observed modulation *in vitro* of the ATPase activity by the purified λ O and P proteins is consistent with such a possibility. Another possibility is that ATP may not serve as the primary substrate for the dnaK protein. Alternatively, the ATPase activity may be necessary for a conformational change of the dnaK protein. The observation that purified dnaK protein is phosphorylated *in vitro* in the presence of [γ-^{32}P]ATP was unexpected. It is not yet certain whether this is due to an autophosphorylation reaction or to trace amounts of a contaminating kinase. The fact that the dnaK protein can be phosphorylated in an anti-dnaK protein IgG precipitate argues in favor of an autophosphorylation mechanism. It is possible that the phosphorylation of dnaK protein represents a trapped enzymatic intermediate of the ATPase reaction. We have observed, however, that a small fraction of the dnaK protein is phosphorylated *in vivo*. This observation, coupled with the fact that phosphorylation results in the formation of phosphothreonine, argues that the phosphorylation of dnaK protein is real and may represent an interesting aspect of the regulation of its activity or of other intracellular enzymatic activities.

Purified dnaK protein, in addition to its ATPase and kinase activities, actively promotes the conversion of M13 single-stranded DNA to a double-stranded form *in vitro* in a crude *E. coli* replication system. This system does not rely on the usual *in vivo* mechanism of M13 single-stranded replication, which is known to require the host *E. coli* RNA polymerase (24). Rather, it is an unusual system in which DNA strand synthesis is initiated in the presence of rifampicin provided that the λ replication proteins O and P are present (unpublished results). We conclude that the dnaK protein, in conjunction with the λ O and P replication proteins and other host replication proteins, participates in the rifampicin-resistant conversion of M13 DNA to the duplex replicative form. The precise molecular role of dnaK protein in this multistep reaction is not known. The dnaK protein could participate in the steps prior to RNA primer synthesis, in the enzymatic steps of RNA primer formation, in the transition from RNA to DNA synthesis, or in the elongation steps of DNA synthesis. The simplicity of this *in vitro* DNA replication system should enable the exact mode of action of the dnaK protein to be determined. Preliminary experiments suggest that the dnaK protein is also essential for λ DNA replication *in vitro* in the more physiological *λdv* replication system of Wold *et al.* (14).

Recently, it has been shown by DNA sequence analysis that the major heat shock protein of *Drosophila*, hsp70, is as much as 50% homologous, at the amino acid sequence level, with dnaK protein (25). Because the hsp70-like proteins of a variety of eukaryotic organisms crossreact immunologically (26), it appears that at least parts of these proteins have been widely conserved during evolution, from bacteria to humans. This conservation has been retained even at the level of protein modification, because a portion of the hsp70 of *Dictyostelium discoideum* has been shown to be phosphorylated at a phosphothreonine residue (27). It would be interesting to determine whether these structural similarities between hsp70 and the dnaK protein have also been conserved at the functional level.

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