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Activity of the Hsp70 chaperone complex—DnaK, DnaJ, and GrpE—in initiating phage λ DNA replication by sequestering and releasing λ P protein

(heat shock proteins/DNA replication/protein–protein interaction)

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ABSTRACT **Initiation of DNA replication by phage λ requires the ordered assembly and disassembly of a specialized nucleoprotein structure at the origin of replication. In the disassembly pathway, a set of *Escherichia coli* heat shock proteins termed the Hsp70 complex—DnaK, DnaJ, and GrpE—act with ATP to release λ P protein from the nucleoprotein complex, freeing the DnaB helicase for its DNA-unwinding reaction. To investigate the mechanism of the release reaction, we have examined the interaction between P and the three heat shock proteins by glycerol gradient sedimentation and gel electrophoresis. We have discovered an ATP-dependent ternary interaction between P, DnaK, and DnaJ; this P·DnaK·DnaJ complex is dissociated by GrpE. We have concluded that the function of the Hsp70 complex in sequestering and releasing P protein provides for the critical step in the disassembly pathway. Based on our data and other work on protein folding, the formation of the P·DnaK·DnaJ complex might involve a conformational shift to a folding intermediate of P.**

Initiation of DNA replication by phage λ requires the ordered assembly and disassembly of a specialized nucleoprotein structure (snup) at the viral origin of replication (oriλ) (1, 2). This sequential pathway serves to localize and "fire" the DnaB helicase of *Escherichia coli*, resulting in an origin-specific DNA-unwinding reaction that provides for the initiation of DNA replication (1, 2). The DnaB helicase is localized at oriλ by the phage O and P proteins. The O protein binds to four direct repeats in oriλ DNA ("iterons"), each of which is an inverted repeat (3, 4); the bound O forms a DNA-wound nucleoprotein structure, the "O-some," which serves as the scaffold for the rest of the assembly reaction (5). The P protein recruits DnaB by forming a P·DnaB complex (6, 7), which binds in turn to the O-some (5, 8–11). The DnaB in the resultant O·P·DnaB snup is inert as a helicase (8–10), presumably because interaction with P quenches the enzymatic activity of DnaB (6, 7).

The intervention of bacterial heat shock proteins disassembles the oriλ snup, freeing DnaB for its DNA-unwinding activity (8–12). The release of P is a characteristic feature of the disassembly pathway and is likely to be the key biochemical event for firing the helicase (9–12). Two disassembly reactions have been characterized with pure proteins: DnaK·DnaJ and DnaK·DnaJ·GrpE. In the more extensively studied DnaK·DnaJ pathway, the two heat shock proteins add to the O·P·DnaB snup, and an ATP-dependent step initiates DNA-unwinding (11) and DNA replication (12, 13). The DnaK, DnaJ, and GrpE proteins act with reduced levels of DnaK to mediate the initiation of DNA replication (10, 12). With the three heat shock proteins, DNA unwinding also occurs efficiently with less DnaK, and the unwinding reaction is more often bidirectional, indicating a more efficient disassembly reaction (C. Wyman, C. Vasilikiotis, D. Ang, C. Georgopoulos, and H.E., unpublished work). Based on a variety of data, the three-protein set of heat shock proteins appears likely to function typically together. DnaJ and GrpE markedly stimulate the ATPase activity of DnaK, the bacterial homolog of the eukaryotic ≈70-kDa heat shock protein Hsp70 (14). Moreover, the three-protein set ("Hsp70 complex") acts together in other reactions, including control of the heat shock regulator σ^{32} (15, 16) and protein folding *in vitro* (17).

In the work reported here, we have sought to understand the protein–protein interactions by which the Hsp70 complex evicts P protein from the initiating nucleoprotein structure. We have identified an ATP-dependent interaction of P, DnaK, and DnaJ that we believe to be the key intermediate in releasing P; this ternary complex is turned over by GrpE to free the individual proteins. We have concluded that DnaB is freed to become active as a helicase ("fired") by the transient sequestering of P (possibly in a partially unfolded conformation). The activity of GrpE provides for a more efficient recycling action of DnaK and DnaJ.

MATERIALS AND METHODS

Proteins. O protein was purified by a modified version of the procedure developed by Roberts and McMacken (18). P protein (19), DnaJ (20), DnaK (21), and GrpE (22) were purified as described. All proteins were highly purified (>90% pure). IgG antibodies to these proteins were raised in rabbits and purified over protein G affinity columns. RNase A was purchased from Sigma.

Glycerol Gradient Sedimentation. Standard 100-μl reaction mixtures contained: 5 μg of P, 8 μg of DnaJ, 21 μg of GrpE, and 56 μg of DnaK (where appropriate) in buffer R [40 mM Hepes·KOH, pH 8.0/11 mM $Mg(OAc)_2$/100 mM KCl]. In standard P reactions, P was incubated with DnaJ or DnaK or both for 5 min at 30°C. ATP was then added, where appropriate, to a final concentration of 2 mM, and the reaction mixture was incubated for another 5 min at 30°C. GrpE was then added, followed by a final incubation at 30°C for 10 min. Total incubation time for all reactions was 20 min at 30°C. In denatured P reactions, P was incubated in 8 M urea at 52°C for 5 min and cooled to room temperature; the denatured P was then diluted 1:20 into solution containing one or more of the heat shock proteins, and incubations were carried out as for native P. Reaction mixtures were then loaded onto 10–30% linear glycerol gradients in buffer R with 5 mM dithiothreitol (preloaded with 5 μg of RNase A as an internal standard). Gradients for reactions with ATP also contained 2

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Abbreviations: Hsp70, ≈70-kDa heat shock protein; snup, specialized nucleoprotein structure; oriλ, phage λ origin of replication.

mM ATP. Centrifugation was carried out for 21 hr at 49,000 rpm with an SW60Ti rotor in a Beckman L65-5B ultracentrifuge. Fractions of 110 μl were collected and analyzed on SDS/15% polyacrylamide gels (19% for gradients containing both P and GrpE). Proteins were visualized by staining with Coomassie brilliant blue R250 (Bio-Rad).

Native Agarose Gel Electrophoresis. Standard 5-μl reaction mixtures contained 50 ng of P, 80 ng of DnaJ, 200 ng of GrpE, and 500 ng of DnaK in buffer R. Proteins were mixed and incubated sequentially as in the sedimentation experiments. Reaction mixtures were loaded onto 0.7% agarose gels in TGE (25 mM Tris base/190 mM glycine/1 mM EDTA) and run at 100 V for 1.5 hr. Proteins then were denatured by soaking the gel in TGS (25 mM Tris base/190 mM glycine/0.04% SDS) for 30 min and transferred onto polyvinylidene fluoride membrane (Millipore) by using a Hoefer Transphor electrophoresis unit. Proteins were detected by immunoblotting with the enhanced chemiluminescence (ECL) detection system (Amersham).

RESULTS

Formation and Turnover of the P·DnaK·DnaJ Complex. *Sedimentation analysis.* The release of P protein from the initiating nucleoprotein structure requires DnaK and DnaJ (8–11). To investigate the mechanism of this release reaction, we have examined the interactions between P, DnaK, and DnaJ by velocity sedimentation in a glycerol gradient. The proteins were mixed at stoichiometries typical of replication reactions, which utilize excess DnaK. The sedimentation position of each protein was determined by polyacrylamide gel electrophoresis of isolated fractions. Relative sedimentation coefficients were calculated with respect to RNase A as a marker protein.

Because the disassembly reaction depends on ATP, we were particularly interested in ATP-dependent interactions. In the presence of ATP, we found no evidence for any two-protein interactions sufficiently stable to persist in the glycerol gradient. To show the sedimentation positions of the individual proteins, an experiment is shown for a mixture of P and DnaK (Fig. 1*A*) and DnaK and DnaJ (Fig. 1*B*) (DnaJ sediments as a dimer). Previous work with affinity column chromatography has shown that DnaK and P interact in the absence of ATP, but the P·DnaK complex is dissociated by ATP (23). We have confirmed this observation by our sedimentation analysis (see Table 1, below). In the absence of ATP, we did not observe an interaction between P and DnaJ or between DnaJ and DnaK (data not shown).

In contrast to the two-protein experiments, we obtained sedimentation evidence for an ATP-dependent three-protein interaction involving P, DnaJ, and DnaK (Fig. 2). Both P and DnaJ exhibited a clear increase in sedimentation velocity (Fig. 2 and Table 1). In addition, some of the DnaK also appeared to sediment more rapidly, although the experiment could not be definitive because of the stoichiometric excess of DnaK (the interaction of DnaK is documented more thoroughly below). The sedimentation increases shown in Fig. 2 do not occur with the nonhydrolyzable analog of ATP, adenosine ($5' \rightarrow O^3$)-1-(thiotriphosphate) (ATPγS) (data not shown). Based on the set of interactions revealed by sedimentation, we have concluded that P, DnaK, and DnaJ form an ATP-dependent ternary complex. Because formation of this complex exhibits the same protein and ATP requirements as the oriλ disassembly reaction, we believe that the P·DnaK·DnaJ complex is likely to be the critical intermediate in the release of P from the initiating nucleoprotein structure.

In addition to DnaJ and DnaK, the GrpE protein is also required for λ replication *in vivo* (24). GrpE interacts with DnaK (22, 25), and GrpE reduces the amount of DnaK needed *in vitro* for efficient DNA replication (10, 12). To investigate the function of GrpE, we examined the influence of this protein on the protein–protein interactions revealed by sedimentation analysis. When GrpE was added to P along with DnaK, DnaJ, and ATP, the rapid sedimentation of P and DnaJ was not observed (Table 1). We have concluded that GrpE acts to turn over the P·DnaK·DnaJ complex, freeing the individual proteins. Thus, in the complete disassembly pathway with DnaK, DnaJ, and GrpE, we presume that P will initially be sequestered by DnaK and DnaJ, and then the set of proteins will be released by GrpE to act again.

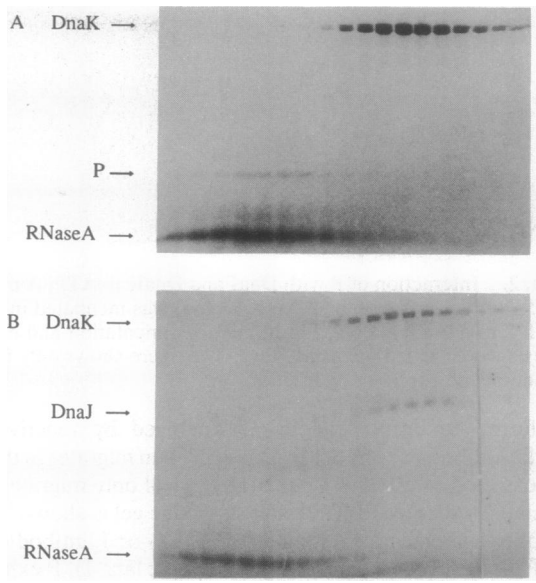

FIG. 1. Lack of two-protein interactions involving P, DnaK, and DnaJ in ATP. (*A*) A mixture of P protein (5 μg) and DnaK (56 μg) in buffer R with 2 mM ATP was incubated at 30°C for 20 min. The reaction mixture was sedimented on a 10–30% glycerol gradient. Fractions were collected, and proteins were fractionated by electrophoresis on 15% polyacrylamide gels. The figure shows the fractions sequentially, starting with the top (most slowly sedimenting) fraction at the far left. Only the top 19 fractions are shown of a total of 38 fractions. (*B*) DnaJ (8 μg) was incubated at 30°C for 20 min with DnaK (56 μg) in buffer R with 2 mM ATP.

To address the specificity of the multiprotein interactions involving P, we have carried out the same series of experiments with λ O protein. We observed an interaction of O with DnaK as noted previously (11); this O·DnaK complex was dissociated by ATP (data not shown). We did not detect any evidence for an ATP-dependent O·DnaK·DnaJ complex (data not shown). Thus, the P·DnaK·DnaJ complex appears to be a specific feature of the disassembly reaction.

Analysis by gel electrophoresis. To confirm the existence of the P–DnaK–DnaJ interaction, we have used agarose gel electrophoresis. Electrophoresis of protein mixtures was carried out under native protein conditions; the proteins were

Table 1. P protein interactions detected by glycerol gradient sedimentation

Protein(s) added	Relative S value	
	P	DnaJ
P alone	1.1	—
DnaJ alone	—	2.0
P + DnaK	1.7	—
P + DnaK + ATP	1.1	—
P + DnaK + DnaJ + ATP	1.7	2.7
P + DnaK + DnaJ + GrpE + ATP	1.1	2.0

Relative S values were calculated with respect to the RNase A internal standard.

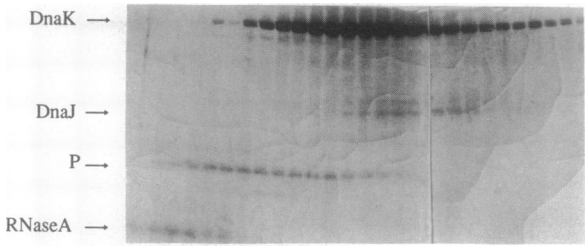

FIG. 2. Interaction of P with DnaJ and DnaK in ATP. A mixture of P (5 μg), DnaJ (8 μg), and DnaK (56 μg) was incubated in buffer R with 2 mM ATP at 30°C for 20 min. Sedimentation and electrophoresis were carried out as in Fig. 1. The figure shows only the top 28 fractions of a total of 38 fractions.

transferred to nitrocellulose and analyzed by reactivity to specific antibodies. The acidic DnaK protein migrates in the gel, whereas the basic P and DnaJ proteins will only migrate when associated with DnaK. The analysis of one gel is shown in Fig. 3, probed either with P antibody (Fig. 3*A*) or J antibody (Fig. 3*B*). P alone failed to enter the gel (Fig. 3*A*, lane 1). P exhibited effective comigration when incubated with DnaK in the absence but not in the presence of ATP (Fig. 3*A*, compare lanes 5 and 6). When P, DnaJ, and DnaK were incubated in the presence of ATP, both P and DnaJ comigrated efficiently with DnaK (Fig. 3, lane 7). This three-protein interaction was largely eliminated in the presence of GrpE (Fig. 3, lane 8). These data confirm the formation of the P·DnaK·DnaJ complex.

In addition to those interactions expected from our glycerol gradient work, we also detected comigration of DnaJ with DnaK in a two-protein reaction (Fig. 3*B*, lanes 3 and 4). An interaction between DnaJ and DnaK has been inferred from genetic experiments (26, 27) and ATPase assays (14); however, direct physical evidence has not been previously obtained. Thus, the agarose gel approach may be highly useful for detecting relatively weak protein–protein interactions.

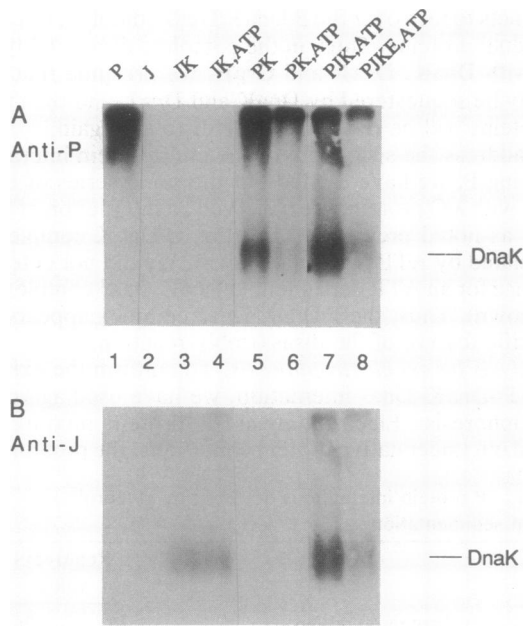

FIG. 3. Interactions detected by native gel electrophoresis. Reaction mixtures containing the proteins indicated were fractionated by electrophoresis in an agarose gel, and proteins were transferred to nitrocellulose membranes and detected by antibody. (*A*) Identification of P protein by anti-P antibody. (*B*) Identification of J protein by stripping and reprobing the *A* membrane with anti-J antibody. Lanes: 1, P alone; 2, DnaJ alone; 3, DnaJ + DnaK; 4, DnaJ + DnaK + ATP; 5, P + DnaK; 6, P + DnaK + ATP; 7, P + DnaJ + DnaK + ATP; 8, P + DnaJ + DnaK + GrpE + ATP.

Formation of Complexes Between DnaJ, DnaK, and Refolding P. Although the P–DnaK–DnaJ interaction has been clearly defined, the special features of the three-protein system responsible for the complex formation have not been identified. Based on other work, we have surmised that DnaJ and DnaK might associate with a partially unfolded P (perhaps a late folding intermediate). In addition to the oriλ disassembly pathway, the DnaK protein and its eukaryotic Hsp70 analog participate in a number of interactions involving unfolded proteins (28, 29). Especially pertinent to the experiments described here, a DnaK·DnaJ·rhodanese complex has been observed as an intermediate in the refolding of denatured rhodanese (17). To investigate interactions with partially unfolded P, we denatured P in 8 M urea and then initiated protein refolding by dilution into buffer with or without the heat shock proteins. By sedimentation analysis, we detected a "refolding P complex" with DnaJ protein not found with native P (Table 2, line 3). A similar complex was not observed with refolding O protein (data not shown). We also observed a three-protein complex involving P, DnaK, and DnaJ (Table 2, line 4). However, the ternary complex was not turned over by GrpE and ATP (data not shown). The data of Table 2 show that DnaJ and DnaK will associate with partially unfolded P, but the complexes formed with native and refolding P are not identical. Possibly a more unfolded form of P is trapped in our artificial experiments than that participating in the native P interactions.

DISCUSSION

Role of the Hsp70 Chaperone Complex in Initiating DNA Replication. The initiation of DNA replication at oriλ results from an ordered assembly and disassembly pathway that localizes and then fires the DnaB helicase. A variety of previous experiments have indicated that the critical biochemical step in the disassembly pathway is the release of P protein from the O·P·DnaB snup by the action of the Hsp70 chaperone complex comprising DnaK, DnaJ, and GrpE (1, 8–12). In this study, we have sought the protein–protein interactions responsible for the release of P. We have identified a ternary interaction of P·DnaK·DnaJ that we believe to be the key intermediate. Formation of this complex requires DnaK, DnaJ, and ATP—the minimal requirements for the disassembly reaction. The ability of DnaK and DnaJ to sequester P might be expected to free DnaB to function as a helicase. The overall disassembly pathway is likely to depend on additional ATP-dependent responses, such as the loss of the O–DnaK interaction in ATP.

The GrpE protein is not required for DNA replication *in vitro* but appears to make the reaction more efficient (10, 12). We have found that GrpE turns over the P·DnaK·DnaJ complex, releasing the individual proteins. In terms of the initiation reaction, we believe that the role of GrpE is to convert a "stoichiometric" sequestering of P protein into a

Table 2. Refolding P interactions detected by glycerol gradient sedimentation

Protein(s) added	Relative S value	
	P	DnaJ
Refolded P alone*	1.1	—
DnaJ alone	—	2.0
dP + DnaJ	1.7	2.3
dP + DnaK + DnaJ	2.3	2.9

Relative S values were calculated with respect to the RNase A internal standard.

*Denatured P apparently refolded completely after dilution in the absence of heat shock proteins, as judged by sedimentation rate in glycerol gradients.

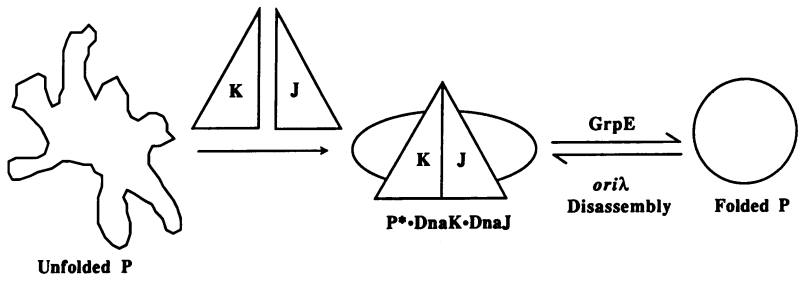

FIG. 4. Proposed mechanism for the action of DnaJ and DnaK. DnaK and DnaJ act to release P protein from the initiating nucleoprotein structure by forming a ternary complex with a partially unfolded conformation of P. The intracellular folding reaction for P protein may be aided by DnaJ and DnaK through association with one or more folding intermediates of P.

more efficient "catalytic" reaction in which P is first sequestered, freeing DnaB, and then the DnaK and DnaJ proteins are released to function again with another origin-bound P protein. We presume that the P protein freed by GrpE cannot inactivate a DnaB helicase in the act of unwinding. Osipiuk and Zylicz have studied the same set of interactions by immunoprecipitation of radioactive P protein (30). They have interpreted their data in terms of an ability of GrpE to strengthen an interaction between a P·DnaJ complex and DnaK. Under experimental conditions that approximate those used in DNA replication, the data of Osipiuk and Zylicz are also consistent with our turnover model. The overall pathway in our proposed mechanism is indicated schematically below:

$$\underset{\text{(DnaB bound)}}{\text{O·P·DnaB}} \xrightarrow[\text{ATP}]{\text{DnaK,DnaJ}} \underset{\text{(DnaB free)}}{\text{P·DnaK·DnaJ}} \xrightarrow{\text{GrpE}} \underset{\text{(DnaB as helicase)}}{\text{P + DnaK + DnaJ.}}$$

Possible Mechanism for P·DnaK·DnaJ Complex—An Unfolding Equilibrium. Although the action of the Hsp70 complex in the oriλ disassembly reaction might be markedly different from other Hsp70 reactions, we consider that a more likely possibility is a fundamentally similar biochemical mechanism based on ability to bind proteins in unfolded conformations (17, 28, 29). The formation of a ternary complex with DnaK and DnaJ has been observed in the folding of denatured rhodanese; moreover, the refolding rhodanese (R*) appears to be trapped in an intermediate conformation between unfolded and native (17). The R*·DnaK·DnaJ intermediate was converted to native rhodanese by the combined action of GrpE and GroEL/ES (Hsp60 complex) (17). Our experiments have shown that refolding P also associates with DnaK and DnaJ.

We believe that an attractive mechanism for the formation of the P·DnaK·DnaJ complex is that depicted in Fig. 4. We presume that native P is in equilibrium with a late folding intermediate P*, with the reaction far toward native P. By means of the P*–DnaK–DnaJ interaction, the equilibrium will shift, and P protein will be sequestered and thereby converted to a conformation inactive for binding to DnaB. The P*·DnaK·DnaJ complex might be a normal intermediate in a general intracellular protein-folding pathway involving the Hsp70 complex (17). The formation of the P*·DnaK·DnaJ complex on the initiating nucleoprotein structure would be driven by the high local concentration of DnaK and DnaJ in the multiprotein complex (10, 11).

The mechanism indicated in Fig. 4 might represent a general pathway for Hsp70-mediated disassembly reactions. For example, DnaK and DnaJ function to activate the RepA protein for binding to the plasmid origin of phage P1 by conversion of dimeric RepA to monomer (31). More generally, the role of the Hsp70 complex in protein folding might involve the rescue of kinetically trapped, misfolded intermediates by a "disassembly" reaction to a more unfolded intermediate able to proceed spontaneously along the correct folding pathway (a type of "editing" function).

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