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A molecular chaperone, ClpA, functions like DnaK and DnaJ

(heat shock proteins/plasmid P1/ATP-dependent proteolysis/ClpP)

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ABSTRACT **The two major molecular chaperone families that mediate ATP-dependent protein folding and refolding are the heat shock proteins Hsp60s (GroEL) and Hsp70s (DnaK). Clp proteins, like chaperones, are highly conserved, present in all organisms, and contain ATP and polypeptide binding sites. We discovered that ClpA, the ATPase component of the ATP-dependent ClpAP protease, is a molecular chaperone. ClpA performs the ATP-dependent chaperone function of DnaK and DnaJ in the *in vitro* activation of the plasmid P1 RepA replication initiator protein. RepA is activated by the conversion of dimers to monomers. We show that ClpA targets RepA for degradation by ClpP, demonstrating a direct link between the protein unfolding function of chaperones and proteolysis. In another chaperone assay, ClpA protects luciferase from irreversible heat inactivation but is unable to reactivate luciferase.**

Molecular chaperones interact with other proteins to mediate ATP-dependent protein folding, refolding, assembly, and disassembly of proteins. The Hsp70 chaperone system of *Escherichia coli* consists of the DnaK, DnaJ, and GrpE heat shock proteins. *In vivo* these three heat shock proteins function together in many cellular processes, as demonstrated by the observations that mutants in *dnaK*, *dnaJ*, and *grpE* have similar effects on DNA replication of *E. coli*, plasmids P1 and F and phage λ, RNA synthesis, cell division, protein transport, regulation of the heat shock response, protection of enzymes from misfolding or aggregation during heat shock, and degradation of abnormal proteins (reviewed in ref. 1). *In vitro* DnaK, DnaJ, and GrpE act in replication of plasmid DNA containing the P1 origin (*ori*P1) (2, 3) and λ origin (*ori*λ) (4–6) and in refolding denatured luciferase (7), rhodanese (8), and RNA polymerase (9, 10).

For *ori*P1 DNA replication, DnaK, DnaJ, and GrpE activate the sequence-specific DNA binding of RepA, the initiator protein. RepA exists as dimers that are inactive for specific DNA binding but form tetrameric complexes with DnaJ dimers (2). DnaK, in a reaction dependent on DnaJ and ATP, activates RepA ≈100-fold for binding *ori*P1 DNA (11). Activation is the conversion of RepA dimers to monomers and the monomers bind with high affinity to *ori*P1 DNA without further help from DnaJ and DnaK (12). GrpE is required for activation at low Mg^{2+} concentrations (≈1 μM free Mg^{2+}) (13). Our model for the mechanism of action of the heat shock proteins in this reaction is that DnaJ tags RepA for recognition by DnaK–GrpE, perhaps by exposing hydrophobic regions. ATP-dependent conformational changes in DnaK cause the dissociation of the RepA–DnaJ–DnaK–GrpE complex. RepA is released as active monomers. Monomers formed by urea denaturation of RepA dimers at low protein concentration followed by dialysis are active for DNA binding, implying that DnaK, DnaJ, and GrpE need not covalently modify RepA (12). Furthermore, monomerization by urea could be reversed by increasing the protein concentration (12).

We have used this well-characterized RepA activation system to identify molecular chaperones and we show below that ClpA is a chaperone. Clp proteins are highly conserved proteins present in all organisms tested, including bacteria [ClpA (14), ClpB (15), ClpX (16, 17), ClpY (HslU) (18)], yeast [Hsp104 (19), Hsp78 (20)], plants [ClpC (14)], hamsters, and humans (19). All have ATP-binding sites and many are heat shock proteins.

E. coli ClpA is a dimer of 84-kDa subunits and assembles into a hexamer in the presence of ATP (21). ClpA–ATP hexamers associate with ClpP, a peptidase sharing no homology with the Clp ATPase family of proteins. The complex, referred to as ClpAP or protease Ti, is active in ATP-dependent degradation of large polypeptides (22–24). Cells lacking ClpA have reduced levels of abnormal protein turnover as do mutants in the other major chaperone genes, including *dnaK*, *dnaJ*, and *grpE*. It has been proposed that chaperones participate in proteolysis by maintaining proteins in unfolded states accessible to proteases. Recently, another member of the Clp family, ClpX, has been shown to activate ClpP for proteolysis (16, 17). The substrate specificities of the ClpAP and ClpXP proteases are different, suggesting the role of Clp ATPase proteins in targeting proteins for degradation.

We show here that ClpA alone functions as a molecular chaperone in the activation of P1 RepA *in vitro*. By characterizing the intermediates and products, we demonstrate the mechanism of this reaction. We also show that ClpA targets RepA for ATP-dependent protein degradation.

MATERIALS AND METHODS

Proteins. Proteins were prepared by procedures slightly modified from those described: RepA (13), DnaJ (13), DnaK (13), GrpE (13), ClpA (25), ClpP (25), and ClpB (26). GroEL and GroES were gifts from J. Buchner (University of Regensburg, Regensburg, F.R.G.), ClpX was from M. Zylicz (University of Gdansk, Gdansk, Poland), bovine Hsp70 and YDJ1 were from E. Eisenberg (National Institutes of Health), human Hsp70 was from R. Morimoto (Northwestern University, Evanston, IL), and yeast Hsp104 was from S. Lindquist (University of Chicago, Chicago).

RepA Activation Reactions. Reaction mixtures (40 μl) for RepA activation contained buffer A (20 mM Tris·HCl, pH 7.5/100 mM KCl/40 mM NaCl/10 mM $MgCl_2$/0.05 mM EDTA/5 mM dithiothreitol), 1 mM ATP, RepA, and chaperones as indicated. After 15 min at 24°C, the mixtures were chilled to 0°C. One microgram of calf thymus DNA and 40

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Abbreviation: ATP[γS], adenosine 5′-[γ-thio]triphosphate.

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fmol of [^{3}H]*ori*P1 DNA fragment were added (11). After 5 min at 0°C, the mixtures were filtered through nitrocellulose filters and the retained radioactivity was measured.

RESULTS

ClpA Substitutes for DnaK and DnaJ in Activating DNA Binding of P1 RepA. In a search for molecular chaperones capable of monomerizing RepA, we assayed known molecular chaperones as well as several potential chaperones in the RepA activation system. We discovered that ClpA activates *ori*P1 DNA binding by RepA (Table 1). Three other Clp proteins, *E. coli* ClpB and ClpX and yeast Hsp104, failed to activate RepA. GroEL and GroES and both bovine and human Hsp70 were also ineffective in activating RepA. Therefore, ClpA acts as a molecular chaperone and there is clear specificity in the interactions of chaperones with substrates.

The 100-fold RepA activation by ClpA is equal to that achieved with DnaJ and DnaK (Fig. 1). ATP (K_a, ≈60 μM) was essential for ClpA activation of RepA (Fig. 1). dATP (1 mM) supported activation by ClpA to the same extent as ATP. It was previously shown that dATP substitutes for ATP for both nucleotide hydrolysis by ClpA and for polypeptide hydrolysis by ClpAP (22). Adenosine 5′-[γ-thio]triphosphate (ATP[γS]), ADP, GTP, CTP, UTP, dGTP, dCTP, or dTTP did not satisfy the ATP requirement. Activation did not occur at 0°C (Fig. 1). However, after activation, RepA was able to bind *ori*P1 DNA rapidly at 0°C. We previously showed that RepA activated by DnaJ and DnaK or by urea bound rapidly to DNA at 0°C (11).

ClpA–ATP Binds RepA and Releases Activated RepA upon ATP Hydrolysis. To determine whether the active form of RepA could be isolated free from ClpA after activation, RepA was incubated with ClpA and ATP and the components were separated by gel filtration chromatography using Sephacryl S-200. Activated RepA, as measured by *ori*P1 DNA binding at 0°C, was efficiently recovered in the included volume of the column (Fig. 2). ClpA was excluded, because it forms a hexamer in the presence of ATP (21). Thus, activated RepA is separable from ClpA and remains activated. In control experiments, when either ClpA or ATP was omitted from the reaction mixture, RepA was not activated but inactive RepA was recovered in the included volume. Gel filtration of a reaction mixture containing RepA and ClpA but without ATP separated the proteins (Fig. 2*B*). RepA eluted at the position of a 70-kDa protein (RepA dimers) and ClpA eluted at the position of a 160-kDa protein (ClpA dimers). Therefore, without ATP, ClpA is not only unable to activate RepA but is also unable to form a complex with it. Similar results were obtained when ATP was replaced with ADP (results not shown).

Table 1. Activation of RepA by molecular chaperones

Protein(s)	[^{3}H]*ori*P1 DNA bound, fmol
1. RepA alone	1.2
2. RepA + DnaJ and DnaK	38.2
3. RepA + DnaJ, DnaK, or GrpE alone	0.9–3.2
4. RepA + ClpA	41.0
5. RepA + ClpB	1.6–3.8
6. RepA + ClpX	0.4–0.8
7. RepA + yeast Hsp104	0.2–0.4
8. RepA + GroEL and GroES	0.6–1.0
9. RepA + bovine Hsp70 ± YDJ1 or DnaJ	0.2–1.1
10. RepA + human Hsp70 ± DnaJ and GrpE	0.2–2.4

RepA activation reaction mixtures contained 30 ng of RepA and the following additional proteins: 1, none; 2, DnaK (0.5 μg) and DnaJ (50 ng); 3, DnaK (0.1–1.0 μg), DnaJ (50–500 ng), or GrpE (50–500 ng); 4, ClpA (360 ng); 5, ClpB (0.2–2.0 μg); 6, ClpX (0.1–0.5 μg); 7, yeast Hsp104 (0.1–2.0 μg); 8, GroEL (0.15–5.0 μg) and GroES (15–500 ng); 9, bovine Hsp70 (0.5–2.0 μg) with or without YDJ1 (100 ng) or DnaJ (100 ng); 10, human Hsp70 (0.2–2.0 μg) with or without DnaJ (100 ng) and GrpE (100 ng).

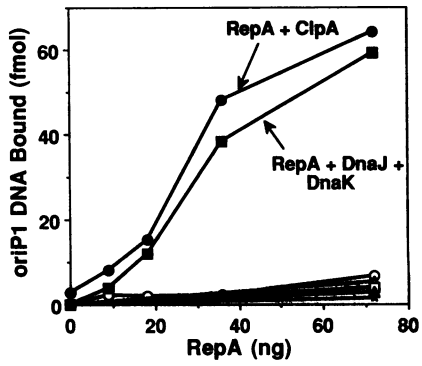

FIG. 1. Activation of RepA by ClpA or DnaJ and DnaK. Reaction mixtures were at 24°C and contained RepA, 360 ng of ClpA, and ATP (●); RepA, 30 ng of DnaJ, 800 ng of DnaK, and ATP (■); RepA and 360 ng of ClpA without ATP (▲); RepA and 360 ng of ClpA and ATP[γS] (□); RepA and ATP (×); RepA, 30 ng of DnaJ, 800 ng of DnaK without ATP (○). Alternatively, mixtures containing RepA, 360 ng of ClpA, and ATP were kept at 0°C (△).

To probe the mechanism of ClpA activation, we sought to demonstrate a complex of the two proteins. RepA and ClpA were both recovered in the excluded volume after gel filtration when the reaction mixture contained ATP[γS] (Fig. 2*C*). The position of the complex was detected by incubating the fractions with an excess of ATP and then measuring DNA binding. Because ClpA was in excess of RepA, all of the RepA was in the RepA–ClpA complex and there was no free inactive RepA. ClpA was quantitatively excluded. RepA alone incubated with ATP[γS] eluted on gel filtration as expected for RepA dimers and was inactive (Fig. 2*D*). ClpA alone incubated with ATP[γS] was excluded by gel filtration, since it forms a hexamer in the presence of ATP[γS] (Fig. 2*E*) (21). We conclude that the ClpA–ATP hexameric structure is required for the interaction of ClpA and RepA. ATP hydrolysis is required for the dissociation of the complex and the release of active RepA.

ClpA Converts RepA Dimers to Monomers. We analyzed RepA activated by ClpA to determine whether ClpA, like DnaJ and DnaK, dissociates RepA dimers into monomers (12). RepA activated by ClpA was chromatographed by gel filtration on Sephacryl S-100, a resin that resolves RepA monomers and dimers. Activated RepA chromatographed as a 33-kDa protein (Fig. 3 *A* and *C*). RepA that had not been activated by ClpA chromatographed as a 68-kDa protein (Fig. 3 *B* and *C*). Furthermore, the electrophoretic mobility on SDS/PAGE of RepA activated by ClpA was indistinguishable from that of 32-kDa RepA subunits. Therefore, ClpA activates RepA by converting RepA dimers to monomers.

ClpA Targets RepA for Degradation by ClpP. Since ClpA activates the ClpP peptidase for ATP-dependent proteolysis (22, 23), we tested whether RepA was a substrate for ClpAP protease. We found that ClpAP degrades RepA, as measured by SDS/PAGE of the products of the reaction (Fig. 4). The products of the reaction determined by C_{18} reverse-phase column chromatography were peptides of <10 kDa. ClpA was also degraded during the reaction. The reaction required ClpA, ClpP, and ATP; dATP but not ATP[γS] satisfied the nucleotide requirement. Consistent with the observation that ClpX could not activate RepA, ClpXP did not degrade RepA (data not shown). Interestingly, RepA bound to *ori*P1 DNA was protected from degradation by ClpAP (Fig. 5*A*). The

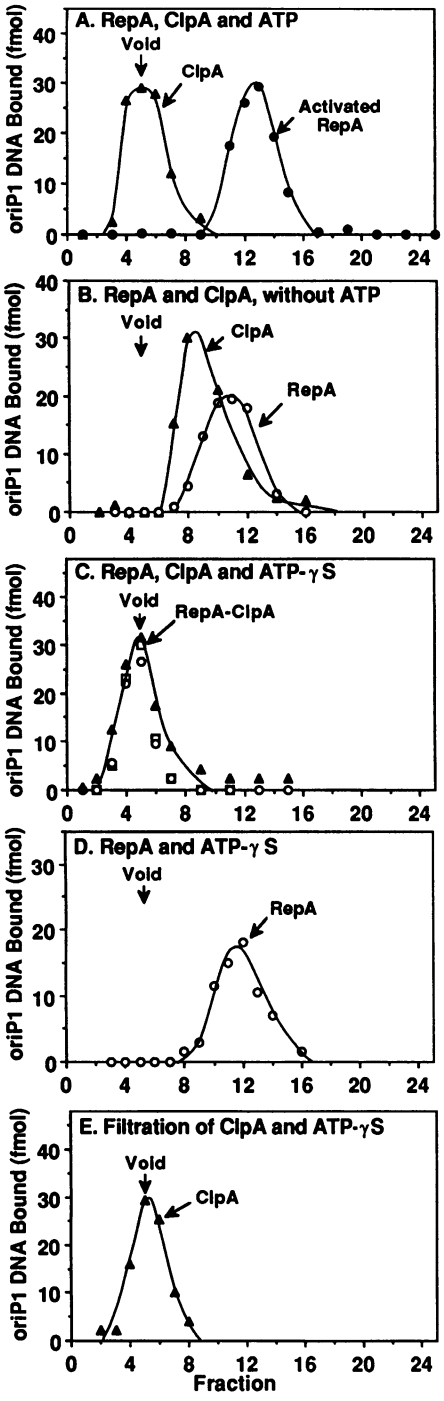

FIG. 2. Isolation of activated RepA and a RepA–ClpA complex. (*A*) ClpA (7.2 μg), RepA (600 ng), and 1 mM ATP were incubated in 400 μl of buffer A for 15 min at 24°C and filtered through a Sephacryl S-200-HR column (0.7 × 18 cm) in buffer A containing 5% (wt/vol) glycerol, 50 μg of bovine serum albumin per ml, and 1 mM ATP. Two milliliters was discarded and fractions (170 μl) were collected. Activated RepA was determined by measuring *ori*P1 DNA binding by 30 μl of each fraction after 5 min at 0°C (●). ClpA was assayed by RepA activation with 30 μl of each fraction and 30 ng of RepA (▲). (*B*) Reaction and filtration were as in *A*, but ATP was omitted. ClpA was assayed as in *A* (▲). RepA was assayed by RepA activation with 30 μl of each fraction, 360 ng of ClpA, 1 mM ATP, and 10 mM $MgCl_2$ (○). (*C*) ClpA, RepA, and 1 mM ATP[γS] were incubated and chromatographed as in *A* but 1 mM ATP[γS] replaced ATP. RepA–ClpA complex was detected by activation reactions with 30 μl of each fraction, 10 mM ATP, and 15 mM $MgCl_2$ (□). ClpA was assayed as in *A* but 10 mM ATP and 15 mM $MgCl_2$ were added (▲). RepA was assayed by RepA activation with 30 μl of each fraction, 360 ng of ClpA, 10 mM ATP, and 15 mM $MgCl_2$ (○). (*D*) Experiment was performed as in *C*, but ClpA was omitted. RepA activity was assayed as in *C* (○). (*E*) Experiment was performed as in *C*, but RepA was omitted. ClpA was assayed as in *C* (▲).

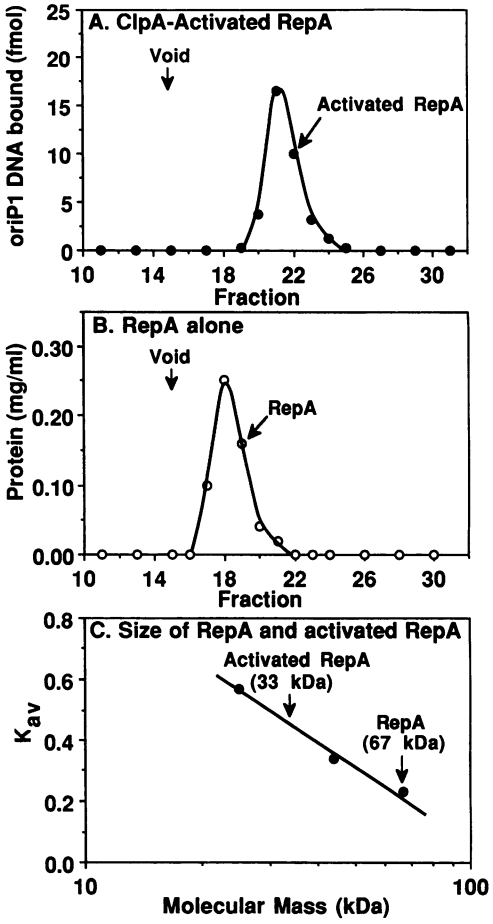

FIG. 3. Gel filtration of RepA before and after activation by ClpA. (*A*) Reaction mixture containing RepA, ClpA, and ATP was incubated and chromatographed as described in Fig. 2*A*, except that a Sephacryl S-100-HR column was used and albumin was omitted. Activated RepA (●) was measured as described in Fig. 2*A*. (*B*) RepA (0.1 mg) in 400 μl of column buffer was applied to the S-100-HR column and protein was determined by the Bio-Rad assay (○). (*C*) Bovine serum albumin (68 kDa), ovalbumin (44 kDa), and chymotrypsinogen (28 kDa) (0.2 mg of each; Pharmacia) were applied separately to the column as in *A* and protein was measured. Elution positions, K_{av} values, were calculated for the protein standards and were plotted as a function of their known molecular masses (●). Arrows indicate observed K_{av} values for RepA (0.23) and activated RepA (0.47).

presence of nonspecific calf thymus DNA did not protect RepA.

ClpA was required for degradation and not simply for monomerization of RepA. We activated RepA by DnaJ and DnaK in a first reaction and then added ClpP or ClpP and ClpA in a second reaction. RepA monomerized by DnaJ and DnaK was degraded in the second reaction only when both ClpA and ClpP were present (Fig. 5*B*). The presence of DnaJ and DnaK did not affect the degradation reaction, even though DnaJ is known to form a tight complex with RepA. Perhaps ClpA and DnaJ recognize different domains of RepA or RepA has a higher affinity for ClpA than for DnaJ.

ClpA Protects Luciferase from Heat Inactivation. Having found that ClpA replaced DnaJ and DnaK in the RepA activation system, we wanted to know whether it could function as a molecular chaperone in another system. We tested whether it could protect and/or reactivate firefly

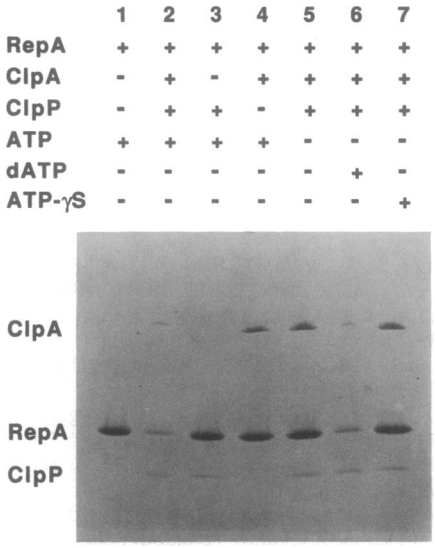

FIG. 4. Degradation of RepA by ClpAP. Degradation reaction mixtures (80 μl) contained buffer A and 1 mM ATP with 3 μg of RepA, 900 ng of ClpA, and 280 ng of ClpP, where indicated. After 40 min at 30°C, proteins were precipitated with 20% (vol/vol) trichloroacetic acid, dissolved in SDS sample buffer, and analyzed by SDS/PAGE.

luciferase from heat inactivation. In this system, DnaJ protects luciferase from irreversible aggregation during heating and DnaJ, DnaK, and GrpE reactivate heated luciferase (7). ClpA with or without ATP was unable to reactivate luciferase when added either before heating or both before heating and again during reactivation (Fig. 6). When present during the heat treatment, ClpA prevented irreversible inactivation of luciferase and DnaJ, DnaK, and GrpE reactivated luciferase (Fig. 6). Therefore, ClpA functions as a molecular chaperone in protecting luciferase from heat inactivation, very likely by preventing its aggregation. ClpA also protects RepA from heat inactivation in the absence of ATP (data not shown). The ATP independence of protection by ClpA implies that it is an activity of dimeric ClpA.

DISCUSSION

Our results suggest the following model for ClpA chaperone function in the activation and degradation of RepA. In RepA activation (Fig. 7), ClpA hexamers bind tightly to RepA dimers only in the presence of ATP, forming a ClpA–ATP–RepA complex. Complex formation, like ClpA hexamerization (21), requires ATP binding but not ATP hydrolysis. ATP hydrolysis causes the release of RepA monomers, very likely due to conformational changes in the ClpA structure. The released RepA monomers bind avidly to *ori*P1 DNA. ClpA hexamers disassemble into dimers because ADP does not stabilize the hexamer structure. RepA activated by ClpA is indistinguishable from that activated by DnaJ and DnaK. Thus, in this system, ClpA performs the ATP-dependent protein remodeling function of DnaJ and DnaK. RepA activation is specific for ClpA or DnaJ and DnaK, since other Clp proteins and other chaperones tested failed to activate RepA.

In the model for RepA degradation (Fig. 7), ClpA specifically targets RepA for degradation very likely through a RepA–ClpA–ATP–ClpP intermediate. It is not known whether RepA recognizes ClpA–ATP–ClpP or ClpP recognizes ClpA–ATP–RepA. By analogy with the mechanism of action of other chaperones, ClpA may unfold RepA, exposing regions that are susceptible to ClpP degradation. Very likely DnaK and DnaJ fail to target RepA for degradation because they do not form a complex with ClpP. This suggests that an

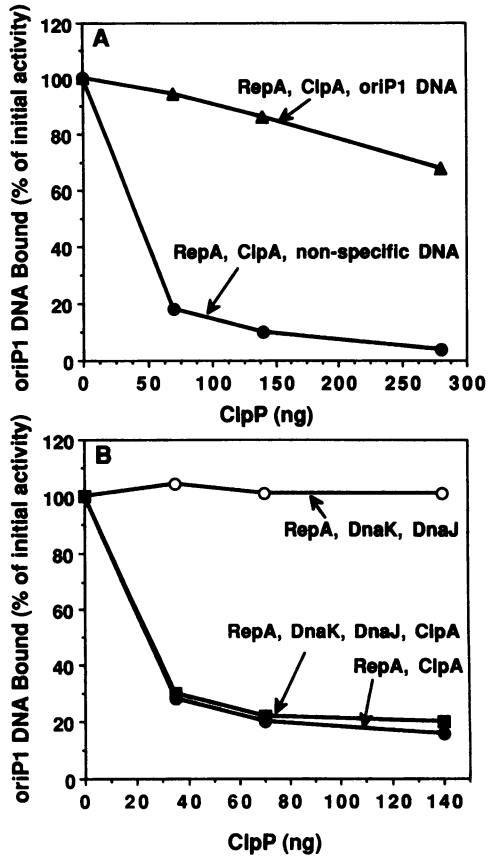

FIG. 5. (*A*) Protection of RepA from degradation by *ori*P1 DNA. Activation reaction mixtures containing 30 ng of RepA and 360 ng of ClpA were incubated for 15 min at 24°C. Reaction mixtures were transferred to 0°C and calf thymus DNA alone (●) or with [^{3}H]*ori*P1 DNA (▲) was added. After 5 min, ClpP was added and reaction mixtures were incubated for 30 min at 30°C. [^{3}H]*ori*P1 DNA was added to reaction mixtures that contained only calf thymus DNA and radioactivity retained on nitrocellulose filters was measured after 5 min at 0°C. It was confirmed by SDS/PAGE that the observed decrease in DNA binding was a quantitative measure of RepA degradation. DNA binding by reaction mixtures without ClpP was 23–25 fmol. (*B*) Requirement for ClpA and ClpP for degradation of RepA activated by DnaJ and DnaK. Activation reaction mixtures containing 30 ng of RepA and 360 ng of ClpA (●); 30 ng of RepA, 30 ng of DnaJ, and 800 ng of DnaK (○); 30 ng of RepA, 30 ng of DnaJ, 800 ng of DnaK, and 360 ng of ClpA (■) were incubated for 15 min at 24°C. ClpP was then added and reaction mixtures were incubated for 30 min at 30°C. After incubation, *ori*P1 DNA binding was measured. DNA binding by reaction mixtures without ClpP was 25–30 fmol after either 15 or 45 min.

ATP-dependent chaperone has to have the ability to form a complex with ClpP peptidase to target proteins for degradation by ClpP.

Our discovery that ClpA has molecular chaperone activity suggests that unfolding and remodeling of target proteins may be an essential energy-dependent step in the selection of substrates and activation of protein degradation by ATP-dependent proteases. The RepA activation/degradation system, in which alternate fates for a single substrate are modulated by its interaction with a single ATP-dependent chaperone, should provide an excellent model for understanding the mechanism of action of other ATP-dependent degradation systems, including the eukaryotic 26S energy-dependent protease (27) and the DnaK-, DnaJ-, and GrpE-dependent degradation of abnormal and heat-aggregated proteins. In both cases, as in the case of ClpAP, target proteins must be specifically selected and refolded to allow either reactivation or degradation.

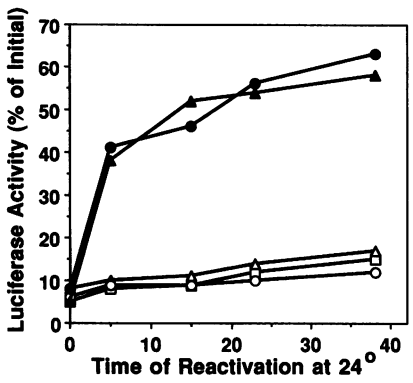

FIG. 6. Protection of luciferase from irreversible heat inactivation by ClpA. Reaction mixtures (50 μl) containing buffer A and 250 ng of firefly luciferase (Sigma) alone or with 900 ng of ClpA or 600 ng of DnaJ, 1 μg of DnaK, and 400 ng of GrpE were heated at 42°C for 10 min. This was followed by incubation at 24°C with 4 mM ATP with or without additional chaperones in the same amounts: no additions to luciferase during heat or reactivation (○); no additions during heat and DnaJ, DnaK, and GrpE during reactivation (□); ClpA during heat and no additions during reactivation (△); ClpA during heat and DnaJ, DnaK, and GrpE during reactivation (▲); DnaJ, DnaK, and GrpE during heat and no additions during reactivation (●). At the times indicated, 3-μl aliquots were withdrawn, diluted 1:100, and analyzed for luciferase activity as described (7).

In summary, we have shown that ClpA has ATP-dependent chaperone activity *in vitro* both alone and as a component of ClpAP protease.

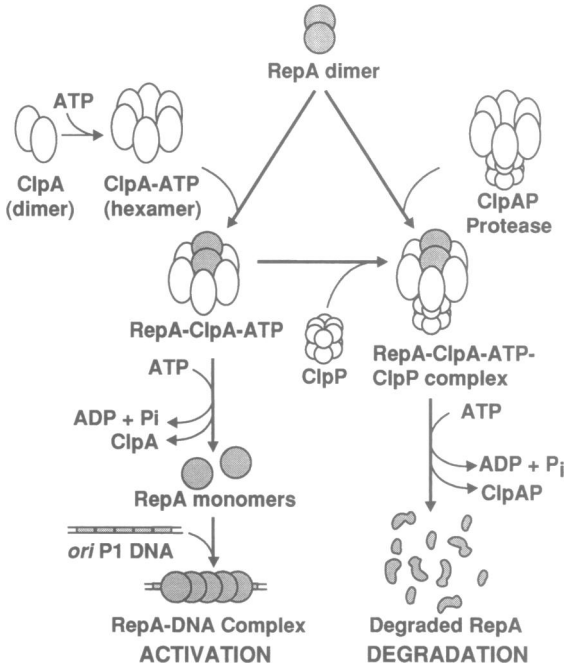

FIG. 7. Model of ClpA function in activation and degradation of RepA. See text for discussion.

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