

Formation In Vitro of Complexes between an Abnormal Fusion Protein and the Heat Shock Proteins from *Escherichia coli* and Yeast Mitochondria

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Heat shock proteins (HSPs) of the Hsp70 and GroEL families associate with a variety of cell proteins in vivo. However, the formation of such complexes has not been systematically studied. A 31-kDa fusion protein (CRAG), which contains 12 residues of *cro* repressor, truncated protein A, and 14 residues of β -galactosidase, when expressed in *Escherichia coli*, was found in complexes with DnaK, GrpE, protease La, and GroEL. When an *E. coli* extract not containing CRAG was applied to an affinity column containing CRAG, DnaK, GroEL, and GrpE were selectively bound. These HSPs did not bind to a normal protein A column. DnaK, GrpE, and the fraction of GroEL could be eluted from the CRAG column with ATP but not with a nonhydrolyzable ATP analog. The ATP-dependent release of DnaK and GroEL also required Mg^{2+} , but GrpE dissociated with ATP alone. The binding and release of DnaK and GroEL were independent events, but the binding of GrpE required DnaK. Inactivation of DnaJ, GrpE, and GroES did not affect the association or dissociation of DnaK or GroEL from CRAG. The DnaK and GrpE proteins could be eluted with 10^{-6} M ATP, but 10^{-4} M was required for GroEL release. This approach allows a one-step purification of these proteins from *E. coli* and also the isolation of the DnaK and GroEL homologs from yeast mitochondria. Competition experiments with oligopeptide fragments of CRAG showed that DnaK and GroEL interact with different sites on CRAG and that the *cro*-derived domain of CRAG contains the DnaK-binding site.

There are two major families of heat shock proteins (HSPs), the Hsp70 and the GroEL families. In eukaryotes, proteins of the Hsp70 family are present in the cytosol (25), lumen of the endoplasmic reticulum (3), and mitochondria (6). Proteins of the GroEL family are found in mitochondria (22), chloroplasts (8), and cytosol of prokaryotes (16). Members of both families have been shown to associate noncovalently with many target proteins and to influence their folding and assembly (26). Hsp70 in eukaryotes (1) and GroEL in *Escherichia coli* bind to nascent polypeptide chains (2). Both can facilitate transport of certain proteins across cell or organelle membranes, apparently by maintaining them in an unfolded state (7, 19). In mitochondria, the GroEL homolog (*hsp60*) binds to proteins transported from the cytoplasm and promotes their folding and assembly into active complexes (23). Also, GroEL was shown to bind to chemically denatured RUBISCO and catalyze its folding and dimerization (13). Recently, in *E. coli*, we found that DnaK, the *hsp70* homolog, and GroEL proteins are present in distinct multicomponent complexes associated with short-lived mutant proteins, such as a nonsecreted mutant *phoA61* polypeptide (26a). The DnaK complex appears to be essential for rapid hydrolysis of this protein, but formation of the GroEL-containing complex does not seem linked to proteolysis (26a). For some reactions, e.g., refolding of a temperature-sensitive *cI* repressor (11) or initiation of lambda DNA replication (27), DnaK requires two additional cofactors, the DnaJ and GrpE proteins, which can bind to DnaK directly in an ATP-dependent manner.

Most of the evidence for the association of HSPs with target proteins has been based on in vivo observations.

However, systematic in vitro studies are essential to clarify the nature of the binding and dissociation reactions and the consequences of complex formation with different HSPs. For example, binding studies with DnaK, GroEL, and model proteins are necessary to define which structural features of a protein are recognized by these two HSPs and whether they bind to the same site, act independently, or require additional cofactors.

To begin to address these important questions, we have studied the interactions of *E. coli* HSPs with an unfolded protein in vitro. We have used a 31-kDa fusion protein (14), which contains 12 amino acids of the *cro* repressor, a truncated protein A, and 14 extra amino acids derived from β -galactosidase at its carboxyl terminus (14). Recently, this hybrid protein, which we refer to by the acronym CRAG, was shown to be associated in vivo with a 70-kDa protein (14), later identified as DnaK (15). We now show that CRAG binds in vivo not only to DnaK but also to GroEL and several minor proteins and that these proteins can be dissociated by ATP. An important advantage of this experimental system is that this hybrid protein, even when complexed to these associated proteins, can still bind to immunoglobulin G (IgG)-Sepharose. Therefore, the binding sites for the HSPs must be accessible when CRAG is associated with IgG. It has been possible to use a CRAG-IgG column as an affinity matrix to isolate the HSPs and to study their association with and dissociation from this abnormal protein.

MATERIALS AND METHODS

Bacterial strains listed in Table 1 were kindly provided by H. Hellebust (Royal Technology Institute, Stockholm, Sweden), J. Beckwith (Harvard Medical School), C. Georgopoulos (University of Utah Medical School), and G. Walker

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TABLE 1. Strains used in this work

Strain	Genotype or phenotype	Source
MPH86	F ⁻ <i>supC</i> (Ts) <i>lac</i> (Am) <i>trp</i> (Am) <i>mal</i> (Am) <i>aro</i> <i>phoA61</i>	J. Beckwith
BB1553	F ⁻ <i>araD139</i> (<i>argF-lac</i>)U169 <i>rpsL150</i> <i>relA1</i> <i>deoC1</i> <i>ptsF25</i> <i>rpsR</i> <i>fbB301</i> <i>dnaK52::Cm^r</i>	G. Walker
MS41	MPH86 <i>dnaK52::Cm</i>	This study
DA15	B178 <i>phoA::Tn10</i>	C. Georgopoulos
DA16	DA15 <i>grpE280</i>	C. Georgopoulos
CG938	C600 <i>dnaJ259</i> <i>minikan^r</i>	C. Georgopoulos
CG2245	B178 <i>groES⁺L⁺ Tn10</i>	C. Georgopoulos
CG2244	B178 <i>groES619 Tn10</i>	C. Georgopoulos
CG2241	B178 <i>groEL44 Tn10</i>	C. Georgopoulos
RR1Δm15	<i>lacZΔm15</i>	H. Hellebust

(Massachusetts Institute of Technology). Cells were grown at 30°C in Luria broth or M9 minimal medium supplemented with vitamin B₁, 0.5% glucose, and all amino acids except methionine. At mid-log phase, the cells were shifted rapidly to 42°C by mixing with an equal volume of the growth medium preheated to 54°C. After 10 min, 0.5 mCi of *trans*-S-label (ICN), a mixture of [³⁵S]cysteine and [³⁵S]methionine (>1,000 Ci/mmol), was added to 100 ml of cell suspension for 20 min to label cell proteins.

The oligopeptides known to bind to *hsp70* (KRQIYTDLE MNRLGKKLIGVLSLFRQK and SNGLQCRIC) were a kind gift from James Rothman (Princeton University). The peptides derived from the N and C termini of CRAG (PGNS RGSVDSQPSL and MQERITLKDYAM) were generously synthesized for us by G. Ramachandran and J. Rosa (Biogen Research Corp.) The purified *groEL* was kindly provided by F.-U. Hartl (University of Munich) and purified *dnaK* by C. Georgopoulos (University of Utah Medical School). The sequencing of the amino terminus of unknown proteins was kindly performed by P. Chao (Biogen Research Corp.).

Preparation of CRAG column. To induce the expression of the CRAG fusion protein, cells of RR1Δm15 strain (Table 1), carrying the pRIT2 plasmid and growing at 30°C in Luria broth, were shifted to 42°C for 2 h because the gene is under the control of P1 promoter, which is regulated by the temperature-sensitive *cI857* repressor. After being harvested, the cells were resuspended in a solution containing 20 mM Tris, 5 mM EDTA, and 1 mg of lysozyme (pH 8.0) per ml, freeze-thawed, disrupted by sonication for 20 s, and then centrifuged at 14,000 × *g* for 10 min. 50 milliliters of the supernatant (1 g of total protein) were applied at 4°C to a 2-ml column of horse IgG-Sepharose (Sigma) and washed with 50 ml of a solution containing 20 mM Tris, 150 mM NaCl, and 1 mM dithiothreitol (pH 7.8). Then DnaK, GroEL, and other proteins associated with CRAG were eluted at room temperature either with 10 ml of 10⁻³ M ATP and 10 mM Mg²⁺ or, in other experiments, with 10 mM Mg²⁺, followed by 10⁻⁵ M ATP plus 10 mM Mg²⁺ and by 10⁻³ M ATP plus 10 mM Mg²⁺ (see below). After elution with ATP, the IgG-Sepharose with bound CRAG devoid of the associated proteins was used in *in vitro* binding experiments.

***In vitro* binding of HSPs to CRAG.** Fifty milligrams of the crude extract of ³⁵S-labeled cells (1 × 10⁶ to 2 × 10⁶ cpm/mg), prepared as described in a previous section, was applied to 2 ml of the CRAG column, washed with buffer until the radioactivity of the eluate had fallen to 5 × 10³ to 8 × 10³/ml, and finally eluted with either 10⁻⁵ or 10⁻³ M ATP

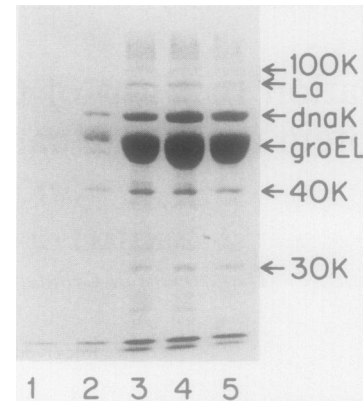


FIG. 1. The association of CRAG *in vivo* with DnaK, GroEL, and other minor proteins. An extract of strain RR1Δm15 which expresses CRAG was loaded onto a 2-ml IgG-Sepharose column, washed with the buffer, and eluted with 10⁻³ M ATP as described in Materials and Methods. Lanes: 1, no ATP; 2 through 5, 1-ml eluted fractions were collected, precipitated with trichloroacetic acid, and analyzed by SDS-PAGE (Coomassie-stained gel).

as indicated. The material which remained bound to the CRAG after elution with ATP was eluted by 100 mM acetic acid, pH 4.5. Under these conditions, most of the remaining labeled proteins were eluted, while CRAG still remained bound to the IgG-Sepharose. The eluted proteins were precipitated with 10% trichloroacetic acid, washed with acetone, and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (18). Western immunoblot analysis was performed as described previously (28).

RESULTS

Binding of *E. coli* HSPs to CRAG. To study the interaction of HSPs with an abnormally folded protein, we used the hybrid protein, CRAG (*cro*-protein A-β-galactosidase) (14). When this fusion protein was first cloned in *E. coli* and isolated, it was reported to be associated with the DnaK protein (15), and this complex could be dissociated by the addition of ATP. Therefore, we prepared an extract from a strain which expressed CRAG, and passed it through an IgG-Sepharose column, as described previously (15). In contrast to the prior results, we found that not only DnaK, but also other proteins were eluted by washing with 10⁻³ M ATP (Fig. 1), including a major protein of 60 kDa and minor components of 100, 90, 40, and 30 kDa. By Western blot analysis with specific antibodies, the 60-kDa protein was identified as GroEL and the 90-kDa was shown to be protease La, the *lon* gene product, which also is a heat shock protein (12). However, in the ATP-eluted fraction, we could not detect with the appropriate antibodies either DnaJ, which in some reactions functions in association with DnaK (20), or GroES, which is generally found associated with GroEL (5). When similar extracts were prepared from MPH86 cells, which did not express any CRAG, no DnaK, GroEL, or protease La was bound to the IgG-Sepharose. Therefore, these proteins do not have inherent affinity for the IgG-Sepharose, and *in vivo* CRAG probably exists in complexes with DnaK, GroEL, protease La, and several unidentified proteins.

After elution of the HSPs with ATP, the CRAG protein remained bound to the column. To test if the bound CRAG could be used as an affinity ligand for HSPs, we loaded a

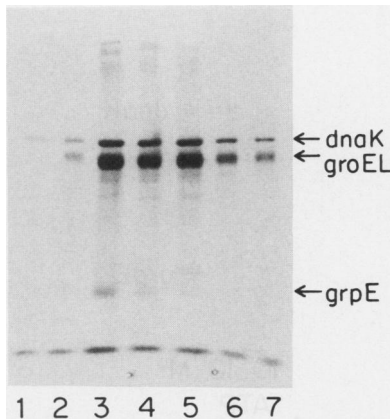


FIG. 2. Binding and release of DnaK, GroEL, and GrpE proteins from CRAG in vitro. Radiolabeled extract from MPH86 cells was loaded on a CRAG-IgG-Sepharose column, washed with buffer, and eluted with 10^{-3} M ATP as described in Materials and Methods. Lanes: 1, no ATP; 2 through 7, proteins eluted with ATP (Fig. 1).

radiolabeled extract of *E. coli*, which does not contain the fusion protein, on the CRAG-IgG column, washed it extensively with buffer, and finally eluted the bound proteins with 10^{-3} M ATP. A small number of proteins were bound and eluted by ATP (Fig. 2), including two major bands, corresponding to DnaK and GroEL, and a minor band of 30 kDa. No protease La, 100- or 40-kDa proteins was found in this eluate, in contrast to eluate from CRAG complexes formed in vivo. To identify the 30-kDa protein, its N terminus was sequenced. A search of standard data base revealed that this sequence matches exactly the known amino terminus of the GrpE HSP. Furthermore, this 30-kDa protein was subsequently shown to react on Western blot with anti-GrpE antibody (data not shown). When a similar cell extract was passed through an agarose column containing wild-type protein A as the ligand, no DnaK, GroEL, or GrpE was bound. Thus, the association of HSPs with CRAG seems to result from its abnormal amino or carboxyl terminus.

Interestingly, not all the bound GroEL and DnaK was eluted with ATP. About 30% of the amount eluted with ATP remained on the column despite repeated washing. This material could be released from the CRAG by washing with acetic acid, pH 2.5. To estimate the portion of total cellular DnaK, GrpE, and GroEL, which bound to CRAG and could be eluted with ATP, we did a Western blot analysis with anti-DnaK, anti-GrpE, and anti-GroEL antibodies of the crude cell extract and of ATP-eluted fractions with 125 I-protein A. (Control experiments with different amounts of extract showed that the amount of 125 I-protein A bound was proportional to the amount of these HSPs present.) We then compared the radioactivity in DnaK, GrpE, and GroEL bands in these two fractions. About 7 to 10% of the total cellular GroEL, about 10 to 15% of the total DnaK, and less than 3% of the total GrpE were eluted with ATP.

If the extract which had passed through the column was reloaded on fresh CRAG columns, no further increase in the amount of these HSPs was found. However, under these conditions, the binding sites on the column are not saturated by DnaK, GroEL, or GrpE. In other experiments, we applied on the same column twice the amount of extract and recovered twice the amount of DnaK and GroEL upon addition of ATP. Thus, only a small fraction of the cellular DnaK, GrpE, and GroEL can bind to CRAG in vitro and be

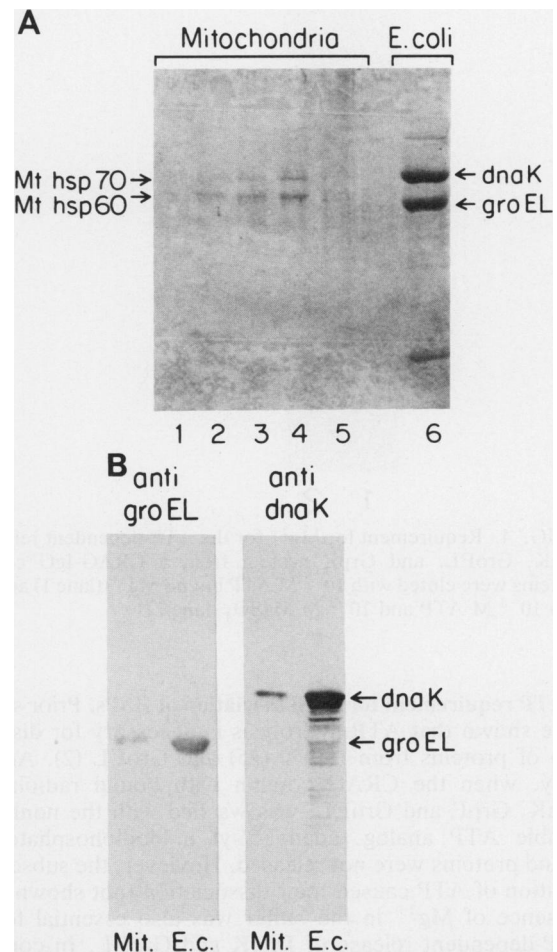


FIG. 3. Binding and release from CRAG of Hsp70 and Hsp60 from yeast mitochondria. Yeast mitochondria were prepared as described by Doum et al. (8) and were lysed with 1% Triton X100 and spun down at $10,000 \times g$ for 10 min. The supernatant was loaded on a CRAG-IgG-Sepharose column, washed extensively with the buffer (see Materials and Methods), and eluted with 1 mM ATP. The eluate was analyzed by SDS-PAGE followed by Coomassie staining (A) and by Western blot with anti-DnaK and anti-GroEL antibodies (B).

eluted with ATP, for reasons that are unclear. Perhaps the remaining DnaK, GrpE, and GroEL in the cell were already bound to other proteins or small ligands and thus were unable to interact with CRAG, although several other explanations are also possible.

Binding of mitochondrial HSPs to CRAG. Mitochondria were recently shown to contain proteins homologous to DnaK (6) and GroEL (22) from *E. coli*. We tested if these mitochondrial proteins behave similarly to the corresponding *E. coli* proteins in their binding to and dissociation from CRAG. An extract from yeast mitochondria was loaded on the *cro*-protein A column, washed, and eluted with ATP. The eluate contained two proteins (evident by Coomassie staining) with molecular masses of 70 and 60 kDa. These proteins reacted on Western blot with antibodies against *E. coli* DnaK and GroEL proteins, respectively (Fig. 3). Thus, these mitochondrial HSPs bind and can be released by ATP in a fashion similar to those of their *E. coli* homologs. Similar results have been obtained with mitochondria from rat liver (17a).

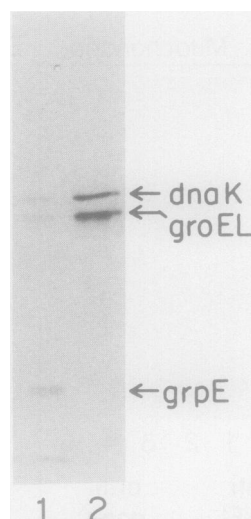


FIG. 4. Requirement for Mg^{2+} for the ATP-dependent release of DnaK, GroEL, and GrpE protein from a CRAG-IgG column. Proteins were eluted with 10^{-3} M ATP but no Mg^{2+} (lane 1) and then with 10^{-3} M ATP and 10^{-2} M $MgSO_4$ (lane 2).

ATP requirement for the dissociation of HSPs. Prior studies have shown that ATP hydrolysis is necessary for dissociation of proteins from Hsp70 (25) and GroEL (2). Accordingly, when the CRAG column with bound radiolabeled DnaK, GrpE and GroEL, was washed with the nonhydrolyzable ATP analog, adenylyl-5'-yl imidodiphosphate, the bound proteins were not released. However, the subsequent addition of ATP caused their dissociation (not shown). The presence of Mg^{2+} in the buffer was also essential for the ATP-dependent release of DnaK and GroEL. In contrast, ATP caused the dissociation of the GrpE protein even without Mg^{2+} present (Fig. 4). Thus, ATP hydrolysis appears necessary for the release of the DnaK and GroEL proteins from the complex with CRAG. Loading of CRAG column with radiolabeled cell extract in the presence of 1 mM ADP and washing with buffer containing 1 mM ADP did not affect the protein composition of subsequent ATP eluate.

Very different ATP concentrations were required for the release of DnaK, GrpE, and GroEL from the complex. Addition of 10^{-5} M ATP released all the DnaK and GrpE but very little or no GroEL, which required 10^{-3} M ATP for elution (Fig. 5). Different concentrations of ATP were then tested to determine the concentrations necessary for elution of these HSPs. The minimal concentration for elution of DnaK and GrpE was 10^{-6} M, while that of GroEL was 10^{-4} M. The minimal concentration of Mg^{2+} for dissociation of all these chaperonins was 10^{-4} M. Thus, dissociation of these proteins from the target can occur independently of one another.

These findings suggest that DnaK and GroEL proteins also bind independently of each other to the hybrid protein. To test this idea, extracts of *dnaK* and *groEL* mutants were used. A radiolabeled extract from a *dnaK* deletion strain (24) (Table 1) was loaded on the CRAG column and was eluted with ATP. As expected, no *dnaK* protein was eluted with ATP; nevertheless, the GroEL protein bound and was eluted in similar amounts as found in the extracts of wild-type cells (Fig. 6). In contrast, we did not find any GrpE in this eluate. Thus, DnaK is not essential for the binding or release of GroEL, but the association of GrpE with CRAG required

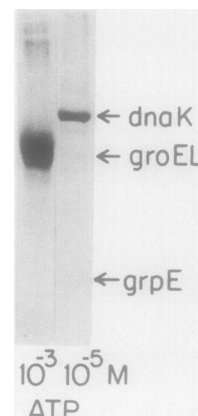


FIG. 5. Different ATP concentrations are required for the release of different proteins from the complex with CRAG. CRAG-IgG column with bound protein was eluted with 10^{-5} M ATP and then with 10^{-3} M ATP.

DnaK. An analogous experiment was done with a ^{35}S -labeled extract from a temperature-sensitive *groEL* mutant. After cells were transferred to the nonpermissive temperature ($43^{\circ}C$ for 20 min) to inactivate GroEL, much less of this protein was bound or eluted than in wild-type cells. Nevertheless, similar amounts of DnaK were bound and released from the column as from extracts of wild-type cells (Fig. 6). Thus, GroEL does not appear to be necessary for the formation of the complex between DnaK and CRAG.

Genetic and biochemical studies have established that the GroES protein interacts with GroEL and that these two proteins work together in promoting protein folding (13). To test whether the GroES protein is involved in the interaction of GroEL with CRAG, a radiolabeled extract was prepared

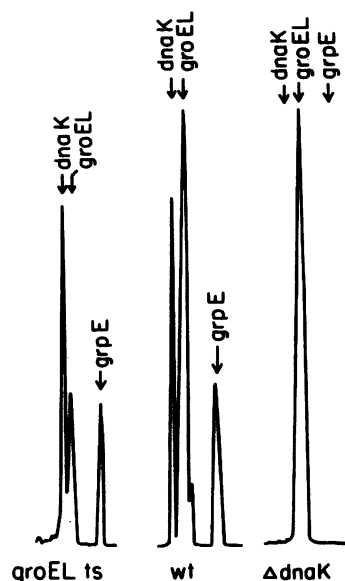


FIG. 6. Effects of a *dnaK* deletion and a *groEL* temperature-sensitive mutation on the binding and release of the DnaK, GrpE, and GroEL proteins from CRAG. Extracts from strains MS41 ($\Delta dnaK$) and *groEL44* and isogenic wild type (wt; middle lane) were used in *in vitro* binding experiments. A densitometric scan of the autoradiograph of the gel is present.

from a temperature sensitive *groES* mutant (CG2244), which was incubated at 43°C for 30 min to inactivate the GroES protein. This treatment did not affect the subsequent binding or release of either GroEL or DnaK (data not shown).

The association of DnaK with the replicative complex of phage lambda is dependent on the DnaJ and GrpE proteins (27). The possible involvement of these proteins in the binding of DnaK to CRAG was examined by using temperature-sensitive mutants in *dnaJ* (CG938) and *grpE* (DA16). Inactivation of these two proteins at the nonpermissive temperatures also did not influence the binding or release of DnaK or GroEL from CRAG (data not shown).

Binding of purified HSPs to CRAG. To test if DnaK, GrpE, and GroEL proteins need additional factors to bind to CRAG, we loaded the IgG column with ³⁵S-labeled extract from the CRAG-containing strain and eluted the bound components with ATP in the usual manner. The eluted fraction containing ³⁵S-labeled HSPs was concentrated to 10 µg of protein per ml and treated with 10 U of apyrase at 30°C for 5 min to hydrolyze the ATP. This fraction was then preapplied to the CRAG column, washed with buffer, and eluted with 10⁻³ M ATP (see Fig. 8). The majority of the purified DnaK and GroEL failed to associate with the column, but a small fraction (3 to 10%) bound and could be eluted with ATP. Under these conditions, no GrpE bound to the CRAG column. Similar experiments with GroEL and DnaK purified by conventional methods (in the laboratories of F. U. Hartl and C. Georgopoulos) also showed that only a small fraction (3 to 5%) of these proteins bound to CRAG. The low amount of binding may possibly indicate that the purified DnaK and GroEL proteins are partially denatured or that they require additional cofactors to associate with target protein.

DnaK and GroEL proteins interact with different sites on CRAG. The experiments were carried out to test whether these HSPs bind to the same domain on CRAG. Since the CRAG protein *in vivo* exists in complexes with DnaK and GroEL proteins, we could release selectively the DnaK protein from the complex with low ATP concentrations and then could test if both DnaK and GroEL could bind to the exposed site(s) *in vitro*. The unlabeled extract from RR1Δm15 was loaded on an IgG-Sepharose column, and the DnaK protein was eluted with 2 × 10⁻⁵ M ATP. At this concentration, the GroEL protein remained bound to the column (Fig. 7I). The column was then loaded with ³⁵S-labeled cell extract, washed with buffer, and finally with 10⁻³ M ATP (Fig. 7II) to release bound DnaK and GroEL. SDS-PAGE of the eluate and radioautography revealed that the only ³⁵S-labeled protein eluted from the column was DnaK. The failure of ³⁵S-GroEL to bind to CRAG under these conditions (Fig. 7III) must mean that the release of DnaK from CRAG freed up binding sites for DnaK but not for GroEL. Thus, DnaK and GroEL must interact with different sites on CRAG (see below).

By using a variant of this approach, evidence was obtained to show that the binding sites for GroEL and DnaK on the cell proteins are different. Purified ³⁵S-HSPs were prepared by elution from the CRAG column with 10⁻³ M ATP. After destroying the ATP with apyrase (as described above), the ³⁵S-DnaK and ³⁵S-GroEL were mixed with a nonradioactive extract from the wild-type strain, and loaded onto the CRAG column. The extract did not inhibit the binding of either ³⁵S-DnaK or ³⁵S-GroEL proteins to CRAG. Thus, these cell extracts must lack protein targets that can compete with the CRAG for binding the ³⁵S-HSPs. Presumably, these endogenous targets are already in complexes with nonradioactive

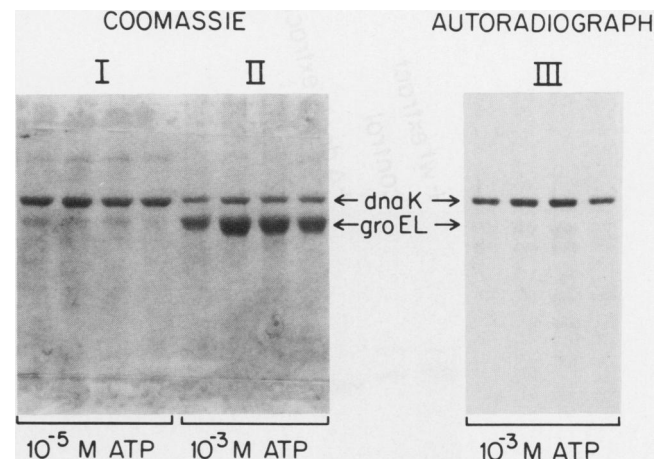


FIG. 7. GroEL does not interact with the DnaK-binding site on CRAG protein. Extract from strain RR1Δm15, which expresses CRAG, was loaded on the IgG-Sepharose column, washed, and eluted with 2 × 10⁻⁵ M ATP to release the DnaK protein from the complex. A radiolabeled extract from strain MPH86 was then loaded on the column, washed, and eluted with 10⁻³ M ATP. Only radiolabeled DnaK bound to the unoccupied binding site.

DnaK and GroEL. In contrast, when an extract from the *dnaK* deletion strain was mixed with the purified ³⁵S-HSPs, it strongly reduced the binding of ³⁵S-DnaK (Fig. 8) without reducing the binding of the labeled GroEL. Apparently, the endogenous binding sites for DnaK were unoccupied in this deletion strain and could compete with the DnaK-binding sites on CRAG. Therefore, in the cell, DnaK and GroEL must bind to different sites on protein.

The DnaK-binding site on CRAG. Finally, an attempt was made to localize the DnaK- and GroEL-binding sites on the CRAG molecule. Since these HSPs do not bind to native protein A, it seems likely that these HSPs bind to the *cro*-derived N terminus or on the β-galactosidase-derived C terminus of CRAG. To test these alternatives, we used the oligopeptides PGNSRGSVDSQPSL and MQERITLKDY AM, corresponding to C- and N-terminal regions of CRAG, respectively, in competition experiments. These oligopeptides in different concentrations were mixed with ³⁵S-labeled cell extract just before loading on the column, and the inhibition of HSPs binding to CRAG was tested. The β-galactosidase-derived oligopeptide, even in concentrations up to 10 mM, did not affect binding of either DnaK or GroEL to CRAG. In contrast, the oligopeptide corresponding to the *cro* region of CRAG strongly reduced DnaK binding at a concentration of 5 × 10⁻⁵ M or higher, but it did not affect GroEL binding (Fig. 9). Thus, the *cro* region of CRAG is important for the binding or recognition of DnaK. The binding of GrpE protein was also inhibited by this peptide, but not as strongly as binding of DnaK. It is also noteworthy that washing the column with 1 mM *cro*-derived oligopeptide did not release the bound DnaK, even though this peptide could prevent the binding reaction. The most likely explanation is that bound DnaK is tightly associated with CRAG, and dissociation requires ATP hydrolysis.

A number of different oligopeptides have been reported to bind *in vitro* to homologs of *hsp70-hsc70* and BIP (10) and to activate the ATPase. Therefore, we tested some of these peptides for their ability to inhibit the binding of DnaK or GroEL to CRAG. At a concentration of 2 × 10⁻³ M, the oligopeptides KRQIYTDLEMNRLGK, KLIGVLSLFR

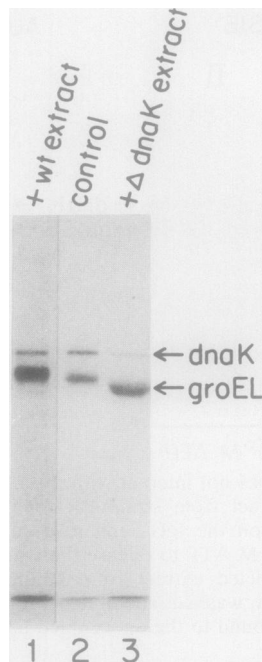


FIG. 8. Inhibition of partially purified DnaK protein binding to CRAG with extract from MS41 ($\Delta dnaK$) strain. ATP-eluted fraction (0.1 ml) containing ^{35}S -labeled DnaK, GroEL, and GrpE proteins was depleted of ATP by apyrase treatment (see Materials and Methods) and mixed with 0.4 ml of the extract from the wild-type or $\Delta dnaK$ strains (1 mg of protein per ml). After incubation for 5 min at room temperature, the extracts were loaded on the column as described in the legend to Fig. 2. The bound material was eluted with ATP prior to electrophoresis. Lanes: 1, no extract added; 2, extract from wild-type strain; 3, extract from $\Delta dnaK$ strain.

PK, and SNGLQCRIC, which bind to Hsp70 (10), caused at most only a minor (15 to 20%) inhibition of binding of both DnaK and GroEL to CRAG. These data confirm the specificity of the interaction of DnaK with the *cro*-derived oligopeptide, which probably contains most of the DnaK-binding site.

DISCUSSION

The present studies introduce a useful experimental system for rapid isolation of the DnaK and GroEL proteins from *E. coli*, as well as the homologous mitochondrial Hsp70 and Hsp60 proteins, and for investigating the association and dissociation of these HSPs from an unfolded polypeptide. In related studies we have also found a novel chaperonin in mammalian cytosol that can bind to this target protein and get eluted with ATP. The properties described here indicate some surprising features of these interactions. For example, the release of proteins associated with GroEL (e.g., RUBISCO) has been reported to require the additional polypeptide, GroES (Hsp10) (13). However, the dissociation of the GroEL protein from the CRAG column occurred in the absence of GroES. Although inactivation of GroES in a temperature-sensitive mutant did not affect the binding or dissociation of GroEL from CRAG, in related studies, we have shown that pure GroES promotes dissociation of the fraction of GroEL that was not released by ATP alone. The requirements for dissociation of GroEL from protein complexes thus depend not only on the nature of the target polypeptides but also on the growth conditions of the bac-

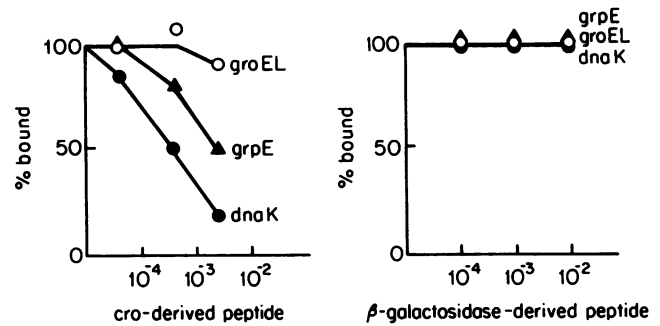


FIG. 9. Inhibition of DnaK and GrpE binding to CRAG with an oligopeptide corresponding to *cro*-derived domain of CRAG. Radio-labeled extract was mixed with different concentrations of the *cro*-derived oligopeptides MQRITLKDYAM (left panel) or the β -galactosidase-derived oligopeptide PGNSRGSVDSQPSL (right panel) and immediately loaded on the CRAG column for in vitro binding experiment as described in the legend to Fig. 2.

teria (26b). In the present experiments, cells were grown routinely at 42°C to raise the level of HSPs in the bacteria. Elsewhere, we shall present evidence that the biochemical requirements for dissociation of these HSP complexes differ in extracts of normal and heat-shocked cells, in which the major HSPs undergo covalent modification (26b).

It is noteworthy that unidentified proteins of 100 and 40 kDa, as well as protease La, were also found complexed to CRAG in extracts of cells and could be released by ATP. The presence of protease La in these complexes suggests a possible role of some of these complexes in degradation of the abnormal protein. In fact, CRAG is a rapidly degraded protein in vivo (26b). Similar complexes were found in our related studies of two other rapidly degraded proteins, the X90 mutant in β -galactosidase and the nonsecreted mutant alkaline phosphatase, *phoA61*. Both these short-lived proteins form in vivo complexes with either DnaK or GroEL proteins plus additional polypeptides (26b), and the complex with DnaK, GrpE, and protease La correlates closely with rapid degradation.

Both DnaK and GrpE proteins could be released from the CRAG complex by 10^{-5} M ATP, while dissociation of GroEL required 10^{-3} M ATP. Thus, this experimental system can be employed for one-step purification of not only DnaK, as suggested by Hellebust et al. (15), but also GroEL from *E. coli* and mitochondrial HSPs. For unknown reasons, these earlier authors did not observe the association of CRAG with GroEL. The presence of the GrpE and protease La might have been missed, since they were generally found in much lower amounts, as are the 100- and 40-kDa polypeptides. GrpE and DnaK proteins have been shown to form complexes with one another both in vivo and in vitro (17, 21). The present findings indicate that the GrpE-DnaK complex can interact with the unfolded protein. Both DnaK and GrpE bound to CRAG and were released together by 10^{-5} M ATP. The *cro*-derived peptide inhibits the binding not only of DnaK but also of GrpE. Furthermore, in the *dnaK* deletion strain, no GrpE was bound to CRAG, despite the overproduction of GrpE in this strain (24). However, we have found that the GrpE from the *dnaK* deletion strain could bind to the CRAG column if some DnaK protein was already associated with CRAG (unpublished data). Thus, the presence of DnaK is necessary for the binding of GrpE to the column. Since DnaK protein tends to form homodimers (21),

complexes with CRAG may be of two forms: GrpE-DnaK-CRAG or GrpE-DnaK-DnaK-CRAG.

One surprising feature of these results is that only a small fraction of the GroEL, DnaK, and GrpE of the cell bound to the CRAG column, even under conditions in which there were many free-binding sites available. Similar results were seen with cell extracts and with purified DnaK and GroEL. A large fraction of these chaperonins may not function *in vitro*, perhaps because additional cofactors are necessary for binding, or because they are partially damaged, or because they are associated with endogenous proteins. Elsewhere we shall present evidence that the fraction of GroEL and DnaK that binds differs from the major species in the cell through some postsynthetic modification (26a).

Although DnaK and GroEL are believed to bind to unfolded proteins selectively, they must recognize different structural features of proteins. For example, extracts from the strain lacking DnaK, which should have many unoccupied DnaK-binding sites, competed with CRAG for binding of ³⁵S-labeled DnaK but not for GroEL. Other competition experiments showed that DnaK and GroEL bind to different regions on CRAG. The selective inhibition of DnaK binding by the N-terminal oligopeptide of CRAG (i.e., by the *cro*-derived region) strongly suggested that this domain includes the DnaK-binding site or recognition site. This inhibitory peptide consisted of 12 amino acids, but the binding site for DnaK could be much smaller. To localize this site more precisely, testing of shorter fragments and genetic modifications of the *cro* sequence of CRAG will be necessary. The failure to inhibit DnaK binding with the carboxyl-terminal sequence of CRAG (derived from β -galactosidase) or with several oligopeptides that bind to Hsp70 and BIP (10) confirms the specificity of this interaction and supports the conclusion that the *cro*-derived region includes the DnaK-binding site. We have not succeeded in identifying the binding site for GroEL, since none of the peptides tested affected GroEL binding. Possibly, GroEL can interact with the junction regions between protein A and β -galactosidase or the *cro* regions of CRAG. In any case, these two HSPs are clearly associating with distinct sequences; presumably, the effects of the association and dissociation of these HSPs also differ in some important fashion.

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