Interaction of the DnaK and DnaJ Chaperone System with a Native Substrate, P1 RepA*

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DnaK, the Hsp70 chaperone of Escherichia coli interacts with protein substrates in an ATP-dependent manner, in conjunction with DnaJ and GrpE co-chaperones, to carry out protein unfolding, protein remodeling, and assembly and disassembly of multisubunit protein complexes. To understand how DnaJ targets specific proteins for recognition by the DnaK chaperone system, we investigated the interaction of DnaJ and DnaK with a known natural substrate, bacteriophage P1 RepA protein. By characterizing RepA deletion derivatives, we found that DnaJ interacts with a region of RepA located between amino acids 180 and 200 of the 286-amino acid protein. A peptide corresponding to amino acids 180–195 inhibited the interaction of RepA and DnaJ. Two site-directed RepA mutants with alanine substitutions in this region were about 4-fold less efficiently activated by RepA, in the region of amino acids 35–49, which interacts with DnaK. An alanine substitution mutant in amino acids 36–39 was constructed and found defective in activation by DnaJ and DnaK. Taken together, the results suggest that DnaJ and DnaK interact with separate sites on RepA.

DnaK, the Escherichia coli Hsp70 homologue, is an ATP-dependent molecular chaperone that acts in conjunction with the co-chaperones, DnaJ and GrpE, to mediate protein folding and remodeling reactions in the cell. The DnaK and Hsp70 chaperone systems participate in a wide variety of cellular processes in both normal and stressed cells, including nascent protein folding, protein trafficking across intracellular membranes, proteolysis, assembly of multiprotein structures, disassembly of protein aggregates, cell division, DNA replication of several plasmids and prophages, and regulation of the heat shock response (see Refs. 1–3 for recent reviews).

Structural studies have shown that DnaK, like other Hsp70s, consists of an N-terminal ATP-binding domain and a C-terminal substrate-binding domain (4, 5). Cycles of ATP binding and hydrolysis by the N-terminal domain modulate peptide binding by DnaK (6–8). In the ATP-bound state, DnaK binds and releases substrates rapidly, whereas in the ADP-bound state, binding and release is slow (9, 10). DnaJ and GrpE regulate cycling between the two states. DnaJ stimulates DnaK ATPase, forming the ADP state of DnaK, which stably interacts with the polypeptide substrate (11). In addition, DnaJ tags several proteins for recognition by DnaK (23, 43). GrpE is a nucleotide exchange factor, binding to the ATPase domain of DnaK and inducing the exchange of bound ADP with ATP (12, 13).

Studies to elucidate the interaction of DnaK and Hsp70 with peptides showed that the chaperones recognize heptameric extended peptides enriched in hydrophobic residues (14–17). Bukau and co-workers (18) performed an extensive study of more than 4000 cellulose-bound peptides spanning sequences of biologically relevant substrates to identify a consensus DnaK binding motif. The motif consists of a hydrophobic core of four to five residues enriched in Leu and to a lesser extent in Ile, Val, Phe, and Tyr, and two flanking regions enriched in basic residues. These sites occur in proteins, not just biologically relevant proteins, on average every 36 residues (18). The DnaJ binding motif was studied by a similar method and found to consist of a hydrophobic core of about 8 residues enriched for aromatic and large aliphatic hydrophobic residues and arginine (19). Although this motif is slightly different from the DnaK binding motif, DnaJ is able to bind most of the same peptides as DnaK (19). Because of the hydrophobic nature of the motifs, DnaK- and DnaJ-binding sites typically reside in the interior of correctly folded proteins and are only exposed in nascent or unfolded polypeptides.

Identification of the DnaJ and DnaK peptide binding motifs helps explain how the chaperone system can interact with unfolded polypeptides. However, in non-stress conditions DnaK protein comprises more than 1% of the total cell protein and is essential for normal growth (20). DnaK would pose a danger to the cell if it indiscriminately unfolded active proteins or disassociated complexes. One of several characterized reactions that the DnaK chaperone system carries out during non-stress situations is the maintenance of plasmid mini-P1 (21, 22). In vitro, DnaK, DnaJ, and GrpE are required for DNA replication of mini-P1 and their sole function is to activate origin-specific DNA binding by RepA by converting inactive RepA dimers to active monomers (23–26). ClpA, a member of the Clp/Hsp100 family of ATP-dependent molecular chaperones, also activates DNA binding by P1 RepA by converting inactive dimers to active monomers (27). Studies to elucidate substrate recognition by ClpA have shown that it recognizes some substrates through N-terminal motifs and others through C-terminal motifs (28–30). RepA is recognized through a motif near the N terminus, between amino acids 10 and 15 (31). To address the question of how the DnaK chaperone system recognizes specific native substrates, we determined regions within a known substrate, P1 RepA, which are recognized by DnaJ and DnaK.

**EXPERIMENTAL PROCEDURES**

Materials—ATP was obtained from Roche Molecular Biochemicals. Restriction endonucleases were obtained from New England BioLabs and polymerase chain reaction reagents were obtained from...
Plasmids—The construction of plasmids expressing N-terminal deletions of RepA, including RepA-(16-286), RepA-(23-286), RepA-(36-286), RepA-(50-286), and RepA-(1-180), was previously described (31). Plasmids expressing C-terminal deletions of RepA were constructed by generating appropriate repA polymerase chain reaction fragments containing 5′ NdeI and 3′ BamHI sites. The fragments were then ligated into pET19b (Novagen), generating appropriate plasmids expressing C-terminal deletions of RepA.

Proteins—DnaJ (32), DnaK (26), GrpE (26), and RepA (23) were purified as described for RepA. N-terminal deletion proteins, RepA(50-286), and alanine substitution mutants were isolated by the method described for RepA. The purity of all proteins was greater than 95% as determined by SDS-PAGE. Protein concentrations were determined by Bradford assay (Bio-Rad) with bovine serum albumin as a standard.

RepA Activation Reaction—Reaction mixtures (20 μl) contained Buffer A (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol (v/v)), 10 mM MgOAc, 1 mM ATP, 100 μg/ml bovine serum albumin, 0.05 pmol of RepA, 0.5 pmol of DnaJ, and 10 pmol of DnaK, unless indicated otherwise. After 10 min at 24 °C, calf thymus DNA (1 μg) and 10 fmol of [3H]oriP1 plasmid DNA were added. The mixtures were then filtered through nitrocellulose filters, the filters were washed, and the retained radioactivity was measured. Activation by ClpA was measured in similar reaction mixtures but with 0.5 pmol of ClpA in place of DnaJ and DnaK.

Co-immunoprecipitation, SDS-PAGE, and Western Blot Analysis—Reaction mixtures (50 μl) contained 1 μM RepA or the RepA derivative, and where indicated, 1 μM DNAJ, and 2 μM DnaK in Buffer A with 5 mM MgOAc, 10 m M MgOAc, 1 mM ATP, 100 μg/ml bovine serum albumin, and where indicated, 1 μM ATP, 100 μg/ml bovine serum albumin.

RESULTS

Recognition of RepA by the DnaJ/DnaK Chaperone System—To investigate the mechanism of native protein recognition by the DnaK chaperone system, we studied the interaction of DnaJ and DnaK with P1 RepA. DnaK and DnaJ activate the latent P1 ori-specific DNA binding activity of RepA by converting inactive dimers to active monomers (24, 25); GrpE is also involved in this process (24, 25).
required under certain conditions to facilitate nucleotide exchange (26).

We tested N-terminal deletion mutants of RepA for their ability to be activated by the combination of DnaK and DnaJ and found that RepA mutants lacking 5, 10, or 15 amino acids were activated as well as wild type RepA (Fig. 1 and data not shown). A deletion missing 25 amino acids was also activated, but to a lesser extent. However, this derivative was also partially defective in DNA binding following activation by treatment with guanidine HCl, suggesting that its defect was not in chaperone recognition but in DNA recognition (Ref. 31 and data not shown). Deletion derivatives of greater than 25 amino acids were not tested because of their impaired DNA binding activity. In contrast, RepA derivatives lacking 15 N-terminal amino acids were not activated by the ClpA molecular chaperone, whereas derivatives lacking 5 or 10 amino acids were activated (Fig. 1B (31)). The results with the derivatives lacking 15 and 25 amino acids indicate that the DnaJ/DnaK chaperone recognition signal in RepA is C-terminal to amino acid 26 and is distinct from the ClpA recognition motif.

Identification of the DnaJ Recognition Site in RepA by Deletion Analysis—To explore the region or regions of RepA recognized by DnaJ, co-immunoprecipitation assays were used to detect DnaJ associated with RepA as described under “Experimental Procedures,” using DnaJ antiserum. C, inhibition of RepA-DnaJ complex formation by RepA-(180–195) peptide. RepA-(180–195) peptide was incubated at the indicated concentrations with DnaJ (50 nM) for 10 min at 24 °C and RepA-DnaJ complex formation was measured as above. Results are mean (± S.E.) of three independent experiments. The apparent K, measured by this assay may not reflect that in solution.

**Fig. 3.** Peptide inhibition of RepA-DnaJ interaction. A, RepA peptide sequences used for inhibition experiments. B, inhibition of RepA-DnaJ interaction by RepA peptides. DnaJ (100 nM) was mixed with various RepA peptides (80 μM), as indicated, for 10 min at 24 °C. Enzyme-linked immunosorbent assay was used to detect DnaJ associated with RepA as described under “Experimental Procedures,” using DnaJ antiserum. C, inhibition of RepA-DnaJ complex formation by RepA-(180–195) peptide. RepA-(180–195) peptide was incubated at the indicated concentrations with DnaJ (50 nM) for 10 min at 24 °C and RepA-DnaJ complex formation was measured as above. Results are mean (± S.E.) of three independent experiments. The apparent K, measured by this assay may not reflect that in solution.
showed that the cell lysates contained RepA or a RepA deletion derivative and DnaJ (Fig. 2B, lanes 1–5). Two species of RepA (1–240) were seen, perhaps the result of degradation. We observed that DnaJ co-immunoprecipitated well with RepA derivatives lacking the C-terminal 26 or 46 amino acids (Fig. 2B, lanes 6 and 7). It co-immunoprecipitated less well with derivatives lacking 66 or 86 amino acids (Fig. 2B, lanes 8 and 9). In contrast, DnaJ was not detected in co-immunoprecipitates with a derivative missing 102 amino acids (Fig. 2B, lane 10). These results suggest that the region in RepA between amino acids 185 and 201 is necessary for the interaction with DnaJ and amino acids in the region of 201–220 contribute to the interaction with DnaJ.

Confirmation of the DnaJ Recognition Site by Peptide Competition—We performed competition experiments using RepA peptides to verify that DnaJ recognized a site in the region of amino acids 185 and 201. For these experiments five RepA peptides were used, corresponding to RepA amino acids 175–189, 180–185, 185–200, 190–206, and 196–211 (Fig. 3A). Wild type RepA was immobilized in microtiter plate wells and then DnaJ alone or DnaJ that had been mixed with one of the RepA peptides was added to the wells. After washing, DnaJ-RepA complexes were detected by enzyme-linked immunosorbent assay. RepA-(180–195) peptide inhibited the interaction of RepA and DnaJ about 90% and 185–200 peptide was inhibited about 20% (Fig. 3B). With this assay, no inhibition by 175–189, 190–206, and 196–211 peptides was detected. A titration experiment showed that 8 μM RepA-(180–195) peptide inhibited the RepA-DnaJ interaction about 50% (Fig. 3C). These results suggest that a DnaJ-binding site resides between amino acids 180 and 200 of RepA.

Site-directed Mutants in the DnaJ-binding Region of RepA Are Defective in DnaJ/DnaK-mediated Activation—A potential problem with the experiments using deletion derivatives was that the proteins may have been misfolded and their lack of interaction with DnaJ may not have reflected the disruption of a true DnaJ-binding site in native RepA. Thus, to substantiate the results of the deletion analysis and peptide inhibition studies, two site-directed mutants were constructed in the region identified by the peptide inhibition experiments. In one mutant, RepA-mut1, alanines were substituted for Tyr-190, Val-192, Leu-193, and Leu-194. In the other, RepA-mut2, Leu-193, Leu-194, His-196, and His-197 were replaced with alanines. We tested the ability of these mutants to be activated by the DnaJ/DnaK system (Fig. 4A). Both mutants were 3–4-fold less efficiently activated for oriP1 DNA binding by DnaJ and DnaK than wild type RepA. In contrast, ClpA activated oriP1 DNA binding by both of the mutants to a similar extent as wild type, suggesting that the mutant proteins are native dimers and can refold into active monomers (Fig. 4B). DNA binding by the mutant proteins was also activated to a similar extent as wild type RepA by treatment with 6 M urea followed by dilution (data not shown). These observations suggest that RepA-mut1 and RepA-mut2 are specifically defective in their ability to be remodeled by DnaJ and DnaK and imply that residues important for recognition are in amino acids 190–197. Because RepA-(190–206) peptide did not appear to interact with DnaJ, it is possible that necessary features of the site are in the region of amino acids 180–190 as well as in the region of 190–197. Taken together, the results of the deletion analysis, the peptide inhibition studies, and the site-directed mutant work indicate that a DnaJ interaction site in RepA is in the region of amino acids 180–200.

Location of a DnaK Recognition Site in RepA—When the immunoprecipitates generated by treating lysates of cells expressing C-terminal RepA derivatives with RepA antiserum (described in Fig. 2B) were probed for DnaK by Western blot analysis, DnaK could be seen associated with all of the C-terminal deletion proteins, although somewhat less appeared to associate with RepA-(1–184), RepA-(1–201), and RepA-(1–220) (Fig. 5A, lanes 4, 6, 8, 10, and 12). In a control experiment, DnaK was not detected when a lysate that did not express a RepA derivative was used (Fig. 5A, lane 2). Thus, DnaK appears to interact with one or more sites in the N-terminal 184 amino acids of RepA. Association of DnaK with RepA-(1–184), which does not interact with DnaJ, indicates that DnaK and DnaJ interact with separate motif/motifs on RepA, although previous work has shown that many peptides that bind DnaK also bind DnaJ (19).

To identify a region in the N-terminal portion of RepA recognized by DnaK, we tested purified N-terminal RepA deletion derivatives in the co-immunoprecipitation assay, using RepA antiserum, for their ability to associate with DnaK. DnaK interacted with RepA derivatives lacking 25 and 35 N-terminal amino acids (Fig. 5B, lanes 2 and 3). Interaction of DnaK with the 25-amino acid deletion mutant substantiated the result that mutant could be activated for DNA binding by the DnaK chaperone system (Fig. 1A). In contrast, no detectable binding was seen between DnaK and a mutant RepA lacking 49 N-terminal amino acids, RepA-(50–286), although that mutant bound DnaJ (Fig. 2A). This result suggested that there is a DnaK-binding site in RepA in the vicinity of amino acids 36–49 (Fig. 5B, lane 4). The sequence in that region is: 3RGLVFVPKPSKGSK29.

We then made an alanine substitution mutant, RepA-mut3, in which Arg-36, Leu-37, Gly-38, and Val-39 were replaced with alanines by site-directed mutagenesis. The mutant was not
detectably activated by DnaJ and DnaK, because the amount of DNA binding seen in the absence and presence of the chaperone system was similar (Fig. 6). The mutant was activated by ClpA, indicating that the structure of the mutant protein was similar to wild type in being recognized by ClpA and converted to active monomers capable of binding DNA (Fig. 6). These results suggest that the alanine substitutions disrupt a necessary recognition signal.

**DISCUSSION**

We have studied the interaction of DnaJ and DnaK with RepA to understand how the DnaK chaperone system recognizes a native protein. The experiments to locate DnaJ recognition sites in RepA by co-immunoprecipitation, peptide inhibition, and site-directed mutation identified a DnaJ-binding site in the region of amino acids 180–200. That 20-amino acid region of RepA contains 13 amino acids expected to be found in peptides that bind DnaJ (19). The peptide that inhibited the interaction of DnaJ and RepA to the greatest extent, RepA(180–195), contains nine residues expected to be found in a DnaJ binding motif within a stretch of 13 amino acids (Fig. 3A). The RepA(185–200) peptide contains nine expected amino acids in a span of 16 amino acids. However, it inhibited RepA-DnaJ binding significantly less well than the 180–195 peptide. The results presented here show that there is a DnaJ-binding site in RepA between amino acids 35 and 49. The probable site in RepA, based on characterization of peptide binding by DnaK by Bukau and co-workers (18), consists of a core of five hydrophobic amino acids between residues 37 and 41, LGVFV, and several flanking basic amino acids, Arg-36, Lys-43, and Lys-46.

Previous work showed that many peptides that bind DnaJ also bind DnaK (19). The observation that DnaK bound RepA with a C-terminal deletion of 102 amino acids but DnaJ did not (Figs. 2B and 5A), suggests that DnaK can bind to a site or sites in RepA distinct from DnaJ sites. The observation that RepA deleted for the N-terminal 49 amino acids bound DnaJ but not DnaK (Figs. 2A and 5B), indicates that the DnaJ-binding site or sites in RepA are specific for DnaJ and not bound by DnaK. It is possible that the sites identified by the deletion analysis reflected hydrophobic regions exposed because of misfolding of the deletion derivatives. However, both peptide competition experiments and site-directed mutations further suggested that these regions are important for recognition by the DnaJ and DnaK chaperone system.

Two mechanisms have been proposed for the targeting of substrates to DnaK by DnaJ (19). In both, DnaJ initially binds to the substrate through specific DnaJ-binding sites. By the first mechanism, the DnaJ- and DnaK-binding sites on the substrate are one and the same. Binding of DnaKATP to the substrate is concurrent with the dissociation of DnaJ from the substrate and the association of DnaJ with DnaK, in a reaction involving DnaJ- and substrate-dependent ATP hydrolysis by DnaJ. In the second mechanism, DnaK associates with a different site on the substrate than DnaJ, generating a ternary complex stabilized by substrate-DnaJ, substrate-DnaK, and DnaJ-DnaK interactions following DnaJ- and substrate-dependent ATP hydrolysis by DnaK. Both models suggest that ADP/ATP exchange, stimulated by GrpE, results in conformational changes in DnaK that cause release of the substrate. Our data supports the second mechanism in which RepA recruits the DnaK chaperone system by specifically and separately interacting with DnaJ and DnaK.

The number of known natural substrates for the DnaK chaperone system is growing rapidly. Many of the substrates identified in vivo have been characterized biochemically, revealing that many are hydrophobic proteins interacting with the bacterial rod. The number of substrates for DnaK and DnaJ is expected to grow as the DnaK chaperone system is studied in more detail.
Substrate Recognition by DnaK and DnaJ

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