Hsp70 family members together with their Hsp40 cochaperones function as molecular chaperones, using an ATP-controlled cycle of polypeptide binding and release to mediate protein folding. Hsp40 plays a key role in the chaperone reaction by stimulating the ATPase activity and activating the substrate binding of Hsp70. We have explored the interaction between the Escherichia coli Hsp70 family member, DnaK, and its cochaperone partner DnaJ. Our data show that the binding of ATP, subsequent conformational changes in DnaK, and DnaJ-stimulated ATP hydrolysis are all required for the formation of a DnaK-DnaJ complex as monitored by Biacore analysis. In addition, our data imply that the interaction of the J-domain with DnaK depends on the substrate binding state of DnaK.

Members of the Hsp70 family, including Escherichia coli DnaK, function as molecular chaperones to mediate protein folding, protein translocation, protein assembly/disassembly, and repair of unfolded proteins damaged by environmental stresses (1, 2). The chaperone activity of Hsp70, with its cochaperone DnaJ (Hsp40 in eukaryotes), involves an ATP-controlled cycle of polypeptide binding and release in which a newly synthesized or translocated polypeptide is promoted to the correct folding by preventing aggregation and misfolding. DnaJ plays a key catalytic role in the chaperone reaction by stimulating the ATPase activity and activating the substrate binding of Hsp70 (3, 4). The kinetics of polypeptide binding and release of Hsp70 are clearly coupled to the Hsp70 ATPase cycle by communication between the ATPase domain and the substrate binding domain of Hsp70 (5, 6). The binding of ATP to the ATPase domain of Hsp70 induces a conformational change in the substrate binding domain of Hsp70 that results in an increase in the on/off rate for a substrate polypeptide and a low binding affinity (5). Subsequent DnaJ-stimulated ATP hydrolysis involves a further conformational change and conversion of Hsp70 to an ADP form that has a slow on/off rate and relatively high binding affinity for a substrate polypeptide (5, 7). Therefore, sequential conformational changes of DnaK during the chaperone cycle play an important role in the regulated activity of DnaK. Previous biochemical studies from partial proteolysis, intrinsic fluorescence, and small-angle x-ray scattering provide evidence that the binding of ATP to the ATPase domain of DnaK induces a subtle conformational change in the ATPase domain followed by a marked conformational change in the substrate binding domain (8–10). However, it is still unclear how the conformational changes of Hsp70 affect DnaJ action.

All DnaJ family members have a J-domain and may also have various combinations of additional conserved and nonconserved regions. E. coli DnaJ consists of two major functional regions. The N-terminal J-domain is primarily responsible for the interaction with DnaK, and the COOH-terminal part containing Gly/Phe-rich, cysteine-rich and less conserved last COOH-terminal regions is involved in the substrate binding (11, 12). The binding of DnaJ to DnaK is dependent on the ATP binding state of DnaK (13). Recently, both genetic and biochemical studies have provided evidence that a conserved tripeptide, His-Pro-Asp, located in the loop between helices II and III of a J-domain, binds in the lower cleft of the DnaK ATPase domain, and some other region of DnaJ binds at or near the DnaK substrate binding site (14, 15). NMR studies using 15N-labeled J-domain also demonstrated that the conserved tripeptide and the residues located in the outer surface of helix II interact with DnaK ATPase domain (16). However, other results indicated that the J-domain alone is not sufficient to stimulate ATP hydrolysis by DnaK (11, 17). J-domain stimulation requires the presence of a DnaK substrate as well (18). This raises the possibility that some region of DnaJ interacts with the substrate binding pocket of DnaK, and we have presented evidence that this is the case (14). However, the region of DnaJ that interacts with the DnaK substrate binding domain and its functional role remains unclear. Because the binding of DnaK to DnaJ is an ATP-dependent process that involves a conformational change of DnaK, this bipartite interaction may control ATP hydrolysis and stabilization of the DnaK-polypeptide complex.

To explore the importance of the conformational changes in DnaK and the bipartite interaction for binding to DnaJ, we analyzed the binding of DnaK point mutants and a series of truncated DnaK polypeptides to DnaJ. We also examined the binding of a series of truncated DnaJ polypeptides and point mutants to DnaK. Our data show that the binding of ATP, subsequent conformational changes in DnaK, and DnaJ-stimulated ATP hydrolysis are all required for the formation of a DnaK-DnaJ complex as monitored by Biacore analysis. In addition, our data imply that the interaction of the J-domain with DnaK depends on the substrate binding state of DnaK.

EXPERIMENTAL PROCEDURES

Materials—Nucleotides, ATP, ADP, ATPγS, and AMP-PNP were obtained from Roche Molecular Biochemicals. Because ADP was contaminated with ATP, ADP was further purified on a HEMA-IEC BIO 1000Q 10U column (Alltech Associates Inc.). DnaJ2–75 protein was generously provided by S. J. Landry (Tulane University).

Expression and Purification of DnaK and DnaJ Proteins—Wild-type or mutant DnaK proteins, T199A and D201N, were expressed and purified as described previously (19). The recombinant genes encoding wild-type or DnaK deletion mutants, K1–403 (pWCS79), K1–538 (pWCS51), K1–628 (pWCS54), R386–638 (pWCS55), and DnaJ dele-
Structural Features Required for DnaK-DnaJ Interaction

RESULTS

The Biacore Assay for Interaction between DnaK and DnaJ—A Biacore assay, which is based on surface plasmon resonance, was used to monitor the protein-protein interactions between DnaJ and DnaK in real time. Surface plasmon resonance detects molecular interactions, because there is a corresponding change in refractive index when a macromolecule in solution binds to a macromolecule immobilized on the sensor chip. Most of our experiments used a DnaJ derivative fused to Bccp at its COOH terminus. This fusion protein supports cell growth of a strain lacking DnaJ, indicating that DnaJ functions are intact in DnaJ-Bccp (data not shown), and permits DnaJ-Bccp to be immobilized via interactions between the biotin group in Bccp and streptavidin coupled to the sensor chip. This coupling avoids the heterogeneity created by directly coupling proteins to the matrix. The high affinity \( K_D = 10^{-15}\) M of the biotin-streptavidin interaction means that there is no significant dissociation of DnaJ during the course of repeated binding experiments.

Consistent with previous results using other assays (13), only the ATP-bound form of DnaK binds to DnaJ immobilized on the sensor chip; the nucleotide-free, ADP-bound, or nonhydrolyzable ATP-bound form of DnaK exhibited no detectable binding (Fig. 1A). Additional validation for this assay comes from our previous results (14) showing that mutants of DnaJ known to be defective in binding to DnaK in other assays also fail to exhibit binding in this Biacore assay, and a mutant DnaJ that restored \emph{in vivo} function to a binding-defective DnaJ variant exhibits binding in this Biacore assay.

The apparent \( k_a \) and \( k_d \) values were determined from linear regression analysis (by plotting \( k_a (\text{slope of the plot of } dR/dt \text{ versus } R) \) against DnaJ concentration) (see “Experimental Procedures”) are \( 1.47 \times 10^4\) M\(^{-1}\) s\(^{-1}\) and \( 8.0 \times 10^3\) s\(^{-1}\), respectively (Fig. 1, B and C). This gives an equilibrium dissociation constant \( K_D = k_d/k_a \) of 544 nm. We previously used a nonlinear least square analysis, which gave a similar \( k_a \) value (\( 2.3 \times 10^4\) M\(^{-1}\) s\(^{-1}\) (14)). However, the \( k_d (= 1.6 \times 10^{-3}\) s\(^{-1}\) obtained from linear regression analysis (\( k_{\text{R}}/k_{\text{A}} \)) of the first 60 data points used previously is 5-fold slower than the \( k_d \) obtained from a secondary plot of \( k_a \) against [DnaJ] (14). As we described recently (14), the residual plots for fitting the DnaK-DnaJ binding curves indicated that the curves fit better to a double exponential than to a single exponential, but the rate constants determined by fitting to a double exponential were irreproducible. The difference in the apparent \( k_d \) values determined by the two methods is likely to result from the usage of different data-subsets from association and dissociation phases, respectively. In view of these problems, we consider this assay simply as a semi-quantitative tool to monitor interaction.

Conformational Changes in DnaK That Result from Binding ATP Are Required for Binding to DnaJ—Both partial proteolysis and intrinsic fluorescence studies have shown that the conformation of DnaK is altered upon ATP binding, raising the possibility that these conformational changes were also required for DnaK to interact with DnaJ. To address this, we examined the binding of DnaJ to the well-characterized DnaK mutant, D201N, which is specifically defective in transmitting the normal conformational change from the active center of the ATPase domain to the substrate domain upon binding ATP as judged by partial proteolysis studies (19). This DnaK mutant is proficient at ATP binding and hydrolysis (19), and a comparable mutation in the ATPase fragment of Hsc70 has very little effect on the ADP-bound structure of the N-terminal ATPase.
Biacore experiments revealed that D201N is defective in binding to DnaJ (Fig. 2A), indicating that the conformational changes lacking in the mutant are required for productive binding.

**ATP Hydrolysis Is Required for the Formation of DnaK-DnaJ Complex**—The lack of DnaK-DnaJ interaction in the presence of ATP analogs, ATPγS or AMP-PNP (Fig. 1A), raised the possibility that ATP hydrolysis may be required to form the stable DnaK-DnaJ complex, which is monitored in the Biacore analysis. However, these data could be explained by the alternative hypothesis that the nonhydrolyzable analogs induced the ADP-dependent rather than ATP-dependent conformation of DnaK, which prevented DnaJ binding (7). To further examine this point, we asked whether the DnaK mutant T199A, which binds ATP and undergoes the same ATP-induced conformation changes as wild-type DnaK but is specifically defective in hydrolyzing ATP (23), can bind DnaJ. We found that T199A exhibited essentially no binding to DnaJ in our conditions (Fig. 2A). These results suggest that ATP hydrolysis is necessary for stable interaction between DnaK and DnaJ with the following caveat. Because the conformational change in DnaK has been monitored only crudely, it remains possible that the binding defect of the mutant results from a slightly altered conformation.

As a second method to examine the hydrolysis requirement, we used results of a recent crystallographic analysis (24) of the nucleotide binding site in the ATPase domain of Hsc70, which revealed that two K\(^{+}\) and one Mg\(^{2+}\) are coordinated with ADP and P\(_{i}\). Functionally, the K\(^{+}\) ions are important for efficient ATP hydrolysis (25, 26). When K\(^{+}\) is replaced with Na\(^{+}\), the steady-state ATPase rates of Hsc70 and DnaK decrease 10- and 3-fold, respectively. We therefore compared the binding of K\(^{+}\)-bound DnaK and Na\(^{+}\)-bound DnaK with DnaJ in the presence of ATP. As predicted if hydrolysis were important, the Na\(^{+}\)-bound form of DnaK is defective in binding to DnaJ relative to the K\(^{+}\)-bound form (Fig. 2B). The peptide-induced stimulation of the DnaK ATPase activity exhibits an even greater dependence on K\(^{+}\) than the basal ATPase, showing an 8-fold preference for K\(^{+}\) over Na\(^{+}\) (26). In the view of this observation, the K\(^{+}\) requirement for DnaJ to bind to DnaK may also result from the necessity for interdomain communication. Taken together, these results are consistent with the idea that formation of the DnaJ-DnaK complex monitored in the Biacore is dependent on ATP hydrolysis as well as the ATP-induced conformational change and interdomain communication. Therefore, the DnaK-
DnaJ binding curve shown in the sensorgram seems to result from the formation of DnaJ-DnaK-ADP complex that is triggered when DnaJ binds to DnaK-ATP.

Features of DnaJ Required for Binding to DnaJ—Genetic and biochemical results have provided evidence that DnaJ interacts with at least two distinct sites on DnaK, the lower cleft of the ATPase domain (14, 15) and at or near the DnaK substrate binding site (14, 27). These results, in conjunction with the requirement of ATP-induced conformational changes for binding of DnaJ to DnaK, raised the possibility that both the ATPase domain and the substrate domain are required for binding to DnaJ. Consistent with this expectation, DnaK variants with only the N-terminal ATPase domain or the COOH-terminal region, including the EEV motif, is important for both the interdomain regulation of Hsp70 function and intermolecular interaction with the DnaJ homolog HDJ-1 (28). The EEVD motif of the eukaryotic Hsp70s corresponds to a conserved EEVD motif in bacteria. The finding that a DnaK variant lacking the COOH-terminal 94 amino acids showed very poor binding of DnaK to DnaJ by using enzyme-linked immunosorbent assay (13) supported this idea. Thus, we further examined whether the last 10-kDa COOH-terminal region, including the EEV motif, is important for interaction with DnaJ. Two deletion mutants, DnaK1–628 (lacking the EEV motif) and DnaK1–538 (lacking the last 10 kDa of the protein) were each examined for binding to DnaJ. Both mutant proteins are capable of binding to DnaJ. DnaK1–628 showed a binding affinity similar to the full-length of DnaK; that of DnaK1–538 is slightly less (Fig. 3B). These in vitro binding results correlate with the in vivo phenotypes of DnaJ binding to DnaK-ATP.

In addition to these two domains, a conserved EEVD motif of the human Hsp70 homolog located in the last 10-kDa COOH-terminal region is important for both the interdomain regulation of Hsp70 function and intermolecular interaction with the DnaJ homolog HDJ-1 (28). The EEVD motif of the eukaryotic Hsp70s corresponds to a conserved EEVD motif in bacteria. The finding that a DnaK variant lacking the COOH-terminal 94 amino acids showed very poor binding of DnaK to DnaJ by using enzyme-linked immunosorbent assay (13) supported this idea. Thus, we further examined whether the last 10-kDa COOH-terminal region, including the EEV motif, is important for interaction with DnaJ. Two deletion mutants, DnaK1–628 (lacking the EEV motif) and DnaK1–538 (lacking the last 10 kDa of the protein) were each examined for binding to DnaJ. Both mutant proteins are capable of binding to DnaJ. DnaK1–628 showed a binding affinity similar to the full-length of DnaK; that of DnaK1–538 is slightly less (Fig. 3B). These in vitro binding results correlate with the in vivo phenotypes of...

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**FIG. 3. Effect of N-terminal or COOH-terminal truncations of DnaK on binding to DnaJ.** A, diagram of the primary structure of DnaK, showing a series of deletion mutants and their in vivo phenotypes. The recombinant genes encoding these deletion mutants that are cloned into the pQE60 vector were transformed into dnaK756 strain and tested for growth at 43 °C and plating efficiency at 30 °C (14). B, each deletion DnaK (1 μM) protein was pre-incubated with 1 mM ATP for 5 min and then injected to allow binding to DnaJ immobilized on a sensor chip.

**FIG. 4. Effect of a truncation of COOH-terminal regions of DnaJ and alanine mutations in the QKRAA motif located at the J-domain on binding to DnaK.** A, binding of DnaJ to various J-domain constructs. The DnaJ76-Bccp, DnaJ108-Bccp, and DnaJ-Bccp fusion proteins were coupled via a biotin group to a streptavidin immobilized on the sensor chip, whereas DnaJ6–6His and DnaJ75 proteins were immobilized on a sensor chip via standard N-hydroxysuccinimide and N-ethyl-N’-(dimethylaminopropyl)carbodiimide activation chemistry. Equal numbers of proteins are immobilized based on both their response unit value (1,000 response units = 1 ng/mm²) and molecular weight of each protein. DnaK (1 μM) was pre-incubated with 1 mM ATP for 5 min and then injected to allow binding to wild-type or deletion mutant DnaJ immobilized on a sensor chip. B, binding of DnaJ to DnaJ alanine substitutive mutants. DnaK (1 μM) proteins were pre-incubated with 1 mM ATP for 5 min and then injected to allow binding to mutant DnaJ immobilized on a sensor chip.

DnaK1–628 and DnaK1–538. Both DnaK(1–628) and DnaK(1–538) support cell growth at 43 °C and growth of bacteriophage λ in cells mutant for dnaK (Fig. 3A). We conclude that the last 10-kDa region including the EEV motif is not required for interaction of DnaJ with DnaK.

Features of DnaJ Required for Binding to DnaK—Previous studies (11, 17) indicated that the minimal unit of DnaJ that stimulates the ATPase activity of DnaK includes both the J-domain and some other portion of DnaJ. Because ATP hydrolysis is required for the binding monitored by the Biacore, the DnaJ domain alone should be unable to bind to DnaK. To test this, we constructed two partial DnaJ proteins, one with the first 76 amino acids of DnaJ (DnaJ76) and a second with the first 108 amino acids of DnaJ (DnaJ108), fused each to Bccp, and then examined binding between immobilized DnaJ deletion mutants and DnaK. The binding affinity of DnaJ108-Bccp to DnaJ in the presence of ATP is comparable to that of full-length DnaJ, whereas DnaJ76-Bccp binds with somewhat lower affinity (Fig. 4A). The binding of DnaJ76-Bccp is specific, because it does not bind to DnaK R167A, which interferes with the interaction between DnaJ and the N terminus of DnaK (data not shown). One possible explanation for the unexpected binding of DnaJ76-Bccp to DnaK is that the Bccp moiety substitutes for some other portion of DnaJ. We therefore tested the ability of DnaK to bind to immobilized DnaJ76 tagged with six...
It has been unclear which portion of DnaJ binds at or near the substrate binding domain, which is required for DnaJ to promote ATP hydrolysis (11, 17, 18). Whereas the conserved J-domain in Sec63 can bind to the substrate binding domain and enhance ATP hydrolysis of its partner protein BIP (4), the native J-domain of DnaJ does not (18). However, addition of the adjacent Gly/Phe region of DnaJ, a COOH-terminal hexa-histidine tag or a fused Bccp protein restores both activities. We consider two possible explanations for this. First, the J-domain of DnaJ may not contain the determinants for binding to the substrate binding domain. In this case, the additional COOH-terminal amino acids may occupy the substrate binding domain of DnaK and stimulate the binding reaction. Although Bccp alone is not a substrate for DnaK, Misselwitz et al. (14) have recently reported that the J-domain of Sec63 broadens the substrate range of BIP. This same factor could permit the Gly/Phe region, a hexa-histidine tag, or Bccp itself to act as substrates to complete the binding reaction. Alternatively, the J-domain, similar to Sec63, may contain such binding determinants but not be in native conformation in the J fragment alone. Addition of COOH-terminal capping amino acids may be necessary to restore proper folding and permit interaction. If the latter explanation were true, the conserved QKRAA motif is a strong candidate for being a portion of the interacting segment, because Auger and Roudier (29) showed that a peptide from the J-domain carrying this motif competed well with all substrate peptides tested for binding to DnaK. We have shown here that the double mutant QAAAA is defective in binding to DnaK. Taken together, it is plausible to consider the possibility that this region of the J-domain interacts with the substrate binding site of DnaK. DnaK substrates with a hydrophobic patch of 4–5 residues and flanked by positively charged amino acids such as Arg or Lys bind preferentially to DnaK (30). The positively charged residues of a substrate peptide seem to interact electrostatically with the negatively charged residues located at the flanking region of the substrate binding pocket. This role could be fulfilled by the Lys-62 and Arg-63 residues in the QKRAA motif.

The finding that DnaJ interacts with the substrate binding domain presents a problem in the actual biological reaction in which substrate is present. There are two issues. First, if DnaJ itself binds to the substrate binding domain, how will authentic substrate binding be facilitated? Second, how are substrates bound to DnaJ targeted to DnaK? Several answers have been proposed, starting with Karzai and McMacken (18), who provided the first evidence that activating the DnaK ATPase requires a bipartite signal involving both binding of substrate and binding of the J-domain. Their suggestion was that DnaJ binding to the substrate binding domain was weak and disso-
associated before that of the J-domain, allowing DnaJ bound substrates to be transferred to DnaK. More recently Misselwitz et al. (14) suggested that interaction of J-domain with either the N-terminal ATPase domain or substrate binding domain of BIP creates a transient open state in BIP for the peptide brought in by DnaJ or associated proteins to bind. Finally Bukau and co-workers (27) have presented evidence that under conditions optimal for folding, a protein substrate in addition to DnaJ is required for ATP hydrolysis and that DnaJ cross-links to the substrate binding domain only in the absence of substrate. Based upon this and other experiments, they argue that the interaction of DnaJ with the substrate binding domain that promotes ATP hydrolysis is an artifact that occurs only at the high DnaJ concentrations used in in vitro binding reactions. Their evidence that the end point interaction monitored in the Biacore is an artifact is compelling. However, we strongly suspect that transient interactions between DnaJ and the substrate binding domain occur during the course of the reaction, based upon the characteristics of the QKRAA motif. Auger and Roudier (29) identified QKRAA, because this motif carries susceptibility to rheumatoid arthritis when expressed on HLA-DRB1. Further studies led them to show that this motif was present in DnaJ and that a QKRAA-containing peptide from DnaJ not only competes all peptide binding to DnaK but also prevents DnaJ binding to DnaK. We have shown that mutating the KR residues decreases DnaJ binding to DnaK. Taken together, we believe that this suggests that the QKRAA region of the J-domain plays a role in the binding cycle, most likely by transiently interacting with the substrate binding domain.

We suggest that DnaJ has two different modes of interacting with DnaK, depending on the situation (Fig. 5). First, DnaJ can interact with a DnaK-substrate complex. Here, conformational changes resulting from binding substrate would facilitate binding to the cleft in the ATPase domain; consequent ATP hydrolysis strengthens the interaction of DnaK with these pre-bound substrates. Second, DnaJ targets substrates to DnaK. Here, the substrate binding site of DnaK would be empty, permitting initial interaction of DnaJ with the substrate binding site of DnaK. This interaction would facilitate the subsequent interaction between the conserved tripeptide in the J-domain and the lower cleft of the ATPase domain; a subsequent conformational change could promote dissociation of DnaJ from the substrate binding domain, allowing the substrate bound to DnaJ to be transferred to DnaK. Only when no substrate peptide is present would DnaJ rebind at the substrate binding site.

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