Molecular chaperones form a class of proteins that bind selectively to nascent, unfolded, misfolded, or aggregated polypeptides and are involved in protein folding, protein targeting to membranes, and protein renaturation after stress. Chaperones, including the DnaK chaperone of *Escherichia coli*, interact specifically with peptides enriched in internal hydrophobic residues, with a preference for positively charged peptides. We previously reported that DnaK interacts with the hydrophobic amino acids Ile, Leu, Val, Ala, Phe, Trp, and Tyr. In the present study, we show that DnaK also possesses a specific binding site for the positively charged amino acids arginine and lysine. Furthermore, the binding of arginine and lysine to DnaK is strengthened when its hydrophobic binding sites are occupied. The specificity of DnaK for Arg/Lys is supported by DnaK-peptide binding studies; the homopolypeptides poly-Arg and poly-Lys interact with DnaK, contrasting with other hydrophilic homopolypeptides, and hydrophobic peptides interact more strongly with DnaK if they contain Arg/Lys at their N terminus. Interestingly, the cochaperone DnaJ attenuates the interaction of DnaK with hydrophobic amino acids while strengthening its interaction with arginine or lysine. The interaction of DnaK with both hydrophobic sequences and with arginine and lysine, and its modulation by DnaJ, may have important implications in both protein folding and protein insertion into membranes.

Chaperones are involved in (a) facilitating the maintenance of other polypeptides in the unfolded state, thus permitting their correct transmembrane targeting, intracellular folding, or oligomeric assembly; (b) the removal of denatured proteins after stress; and (c) the disassembly of several protein complexes (reviewed in Refs. 1–3). One class of chaperones is formed by the 70-kDa heat shock proteins (hsp70), which includes DnaK protein of *Escherichia coli* (4, 5). DnaK is involved in protein folding, protein secretion, lambda phage replication, and regulation of the heat shock response (1–3). DnaK has also been implicated in cell division (6), murine synthesis (7), flagellar assembly (8), and osmoregulation (9). Members of the hsp70 family can distinguish native proteins from their non-native forms (4, 10), owing to the specificity of their peptide binding site. They reversibly bind unfolded proteins and peptides with concomitant ATP hydrolysis and display a peptide-dependent ATPase activity (4, 10–12). Several studies with BIP, DnaK, and Hsc70 have shown that chaperones interact specifically with peptides enriched in internal hydrophobic residues, with a preference for positively charged peptides (12–15). BIP binds hydrophobic peptides (12, 15). DnaK binds peptides containing hydrophobic residues and terminal positively charged residues (13). Hsc70 binds peptides containing hydrophobic residues and basic amino acids, predominantly lysines (14). We have recently reported that DnaK interacts specifically with the aliphatic amino acids (Ile, Leu, Val, Ala) and the aromatic amino acids (Phe, Trp, Tyr) (16). In the present study, we show that DnaK interacts specifically with the positively charged amino acids arginine and lysine, and we describe the effect of DnaJ on the hydrophobic sites and on the Arg/Lys binding sites of DnaK. The Arg/Lys binding site of DnaK possibly interacts with the polar head of hydrophobic sequences and may be involved in protein folding, protein renaturation (14, 17–20), and protein targeting to membranes (21, 22).

EXPERIMENTAL PROCEDURES

Purification of DnaK and DnaJ—DnaK and DnaJ were prepared, as described previously (23–25), from an overproducing strain of *E. coli* bearing plasmid pLNA2 derived from plasmid pDM38 (26) (a gift from Dr. O. Fayet, Microbiologie et Génétique Microbiennue CNRS, Toulouse, France).

Size Exclusion Chromatography—For DnaK-peptide binding assay, a HPLC gel permeation column (TSK G2000SW, fractionation range 500–60,000 Da) was equilibrated with 50 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM Tris-2-mercaptoethanol, 100 μg/ml bovine serum albumin. 10-μl reaction mixtures, containing DnaK (DnaJ and ATP as indicated), radiolabeled unfolded bovine pancreatic trypsin inhibitor (BPTI), or radiolabeled reduced-carboxymethylated lactalbumin (R-CMLA), and competing peptides (labeled substrate proteins and competing peptides were mixed before addition of DnaK) were incubated for 30 min at 23 °C in the same buffer without serum albumin and applied to the column at room temperature. Fractions were collected at a flow rate of 1 ml/min and counted for radioactivity. Unfolded BPTI was prepared as described previously (16), and R-CMLA was obtained from Sigma. Both proteins were labeled by the chloramine-T method (10).

ATPase Assay—The ATPase reaction mixture was incubated at 23 °C and contained the following components in a volume of 3 μl: 20 mM Tris-HCl, pH 7.4, 2 mM KCl, 1 mM Tris-2-mercaptoethanol, 50 μM [3H]ATP (1.5 Ci/mmol), 100 μM MgCl₂, 0.2 μM DnaK (and 0.2 μM DnaJ, when indicated), and amino acids or peptides as indicated. The reaction was linear as a function of time, and ADP production was terminated by applying 2 μl of the sample to polycrylamide cellulose thin-layer chromatography plates that had been spotted with carrier nucodide as described in Ref. 11. A relative activity of 1 (as plotted in the figures) represents 1.9 nmol of ADP released/min/mg of DnaK.

Materials—ATP disodium salt was from Sigma. [3H]ATP was obtained from Amersham and was used at 1.5 Ci/mmol. L-Amino acids were used in solutions adjusted to pH 7.4. Substance P and its derivatives were obtained from Bachem Feinchemikalien AG (Switzerland), and poly-L-amino acids were obtained from Sigma.

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed. Tel.: 33-1-44-27-50-98; Fax: 33-1-44-27-35-80.

‡The abbreviations used are: HPLC, high pressure liquid chromatography; BPTI, bovine pancreatic trypsin inhibitor; R-CMLA, reduced-carboxymethylated lactalbumin.
RESULTS

Stimulation of the DnaK ATPase by Arginine and Lysine—As shown in Fig. 1A, both arginine and lysine stimulate the DnaK ATPase at concentrations higher than 5 mM, a phenomenon that we did not test thoroughly in our previous study (16). Despite the high amino acid concentrations required for DnaK stimulation, we believe that this interaction is significant for the following reasons: (i) the other hydrophilic or charged amino acids (except threonine (16)) do not stimulate DnaK even at high concentrations (not shown); (ii) the stimulation of DnaK by arginine or lysine occurs at lower concentrations (K_a = 2 mM) in the presence of amino acids that can bind to the hydrophobic sites of DnaK, for example 3 mM leucine (Fig. 1A) or isoleucine (not shown) (hydrophobic amino acids do not potentiate the interaction between DnaK and other hydrophilic amino acids (not shown)); (iii) the specificity of DnaK for Arg/Lys is supported by peptide binding studies (see below and Ref. 13). Thus, DnaK seems to possess a specific Arg/Lys binding site whose affinity is increased when the hydrophobic sites of the chaperone are occupied.

Interaction of Arginine and Lysine with the Peptide Binding Site of DnaK—To ascertain that arginine and lysine exert their effect by interacting with the peptide binding site of DnaK, we studied their ability to compete for protein binding to DnaK. The binding of unfolded BPTI to DnaK, measured by coelution of 125I-BPTI (1 μM) with DnaK (1 μM) on a gel permeation column is inhibited by arginine or lysine (K_a = 3 and 4 mM, respectively) (Fig. 1, B and C). The binding of R-CMLA to DnaK is also inhibited by arginine and lysine (see below, Fig. 4C). These results suggest that arginine and lysine interact with the peptide binding site of DnaK.

Interaction between DnaK and Poly-Arg or Poly-Lys—The specific interaction between arginine/lysine and DnaK is supported by an interaction between the homopolymer peptides poly-Arg and poly-Lys and the chaperone. Poly-Arg (75 residues) and poly-Lys (75 residues) can, respectively, stimulate 3- and 2-fold the DnaK ATPase (K_a = 6 and 2 μM) respectively, while other hydrophilic homopolymer peptides of similar length, poly-Glu, poly-His, and poly-Asp (not shown), have no effect on the DnaK ATPase. Furthermore, the binding of unfolded BPTI to DnaK is inhibited by poly-Arg and Poly-Lys (K_a = 16 and 8 μM respectively) (Fig. 2B). This result suggests that DnaK can interact with Arg/Lys-sequences containing sequences without requiring the presence of neighboring hydrophobic amino acids in the sequence. Hsc70 interacts with arginine/lysine-rich sequences of karyophilic proteins (such as PKKKRKV of SV40 T-antigen) for their nuclear import (14). The SecB chaperone of E. coli also interacts with positively charged amino acids, and this interaction is believed to facilitate the exposure of its hydrophobic sites (27).

Interaction between DnaK and Hydrophobic Peptides Containing Arg/Lys at the N Terminus—We investigated the roles of hydrophobic amino acids and arginine/lysine in DnaK-peptide interactions by using three peptides with a hydrophobic C terminus, two of them containing Arg/Lys at the N terminus (R/K in the one letter code). Substance P (RPKQFQFFGLM) and substance P2-11 (PKPQFQFFGLM), which both contain Arg or Lys at their N terminus, interact more strongly with DnaK than substance P4-11 (POQFFGLM); they stimulate the DnaK ATPase at lower concentrations (K_a = 10, 15, and 45 μM, respectively) (Fig. 3A), and they inhibit more efficiently the binding of unfolded BPTI to DnaK (half-maximal inhibitory concentration = 15, 20, and 80 μM, respectively) (Fig. 3B). Thus, the presence of a positively charged amino acid (Lys of substance P) strengthens peptide binding to DnaK (compare P2-11 and P4-11), while the presence of a second positively

![Image](http://www.jbc.org/)

**Fig. 1. Interaction between DnaK and arginine or lysine.** A, stimulation of the DnaK ATPase. The DnaK ATPase was measured as described under "Experimental Procedures" at the concentration of Arg (●) or Lys (□) indicated in the abscissa, in the absence (open symbols) or in the presence (closed symbols) of 3 mM Leu. Each point represents the mean value of three experiments. A relative activity of 1 represents the unstimulated activity of DnaK, which amounts to 1.9 nmol ADP/min/mg of protein. The vertical bars give estimates of the errors. B, binding experiments. Radiolabeled unfolded BPTI (1 μM) was preincubated with DnaK (1 μM) for 30 min in the absence (○) or in the presence of 5 mM Arg (●), or 5 mM Lys (▲), and chromatographed on a HPLC TSK G2000SW gel permeation column as described under "Experimental Procedures." Fractions were collected and counted in a scintillation counter. C, radiolabeled BPTI (1 μM) and DnaK (1 μM) were incubated in the presence of arginine (○) or lysine (□) at the final concentrations indicated in the abscissa and chromatographed as described above. The results are expressed as the amount of BPTI bound to DnaK in the presence of arginine or lysine, relative to the amount bound in the absence of amino acids.
charged amino acid (Arg 1 of substance P) leads to an only slight additional increase in binding (compare P and P 2–11). These results suggest that the presence of Arg/Lys at the N terminus of hydrophobic peptides is beneficial, although not essential, for their interaction with DnaK and is in accordance with DnaK-peptide binding studies performed with a peptide display library (13).

Effect of DnaJ on the Hydrophobic Binding Sites and on the Arginine/Lysine Binding Site of DnaK—In a recent study, we showed that the GroEL's preference for hydrophobic amino acids is reversed by GroES to hydrophilic amino acids, and we have suggested that a hydrophobic/hydrophilic flip flop of the GroEL/GroES chaperone machine might be involved in its function (28). The following results suggest that, in a similar manner, DnaJ attenuates the hydrophobic sites of DnaK while strengthening its arginine/lysine site.

In the absence of DnaJ, isoleucine stimulates the DnaK ATPase (as reported in Ref. 16). In the presence of DnaJ, the DnaK ATPase is stimulated severalfold by DnaJ, as reported in Ref. 29, and isoleucine does not overstimulate the DnaK ATPase (Fig. 4 A). Similar results were obtained with Phe (not shown). In contrast, arginine stimulates the DnaK ATPase more strongly in the presence of DnaJ than in its absence (Fig. 4B) (similar results were obtained with lysine (not shown)). Thus, while DnaJ might counteract the interaction between hydrophobic amino acids and DnaK, it appears to stimulate the interaction between arginine/lysine and the chaperone.

This conclusion is supported by the following binding experiments. Arginine, lysine, isoleucine, and phenylalanine were tested for their ability to compete for R-CMLA binding to DnaK either in the absence or the presence of DnaJ and/or ATP (R-CMLA was chosen for this study because it interacts with DnaK, but not significantly with DnaJ (30)). The binding of R-CMLA to DnaK was measured by coelution of radiolabeled R-CMLA with DnaK on a HPLC gel permeation column in the presence of competing peptides, relative to the amount bound in their absence.
relative efficiencies of the amino acids in competing for R-CMLA binding to DnaK were not significantly altered (not shown)). In the presence of DnaJ and ATP, the addition of 10 mM isoleucine or phenylalanine has no effect on the binding of R-CMLA to DnaK, whereas the addition of 10 mM arginine or lysine reduces R-CMLA binding to DnaK to 28 and 22%, respectively (Fig. 4C). These results suggest that, in the presence of DnaJ, the DnaK-peptide interaction shifts from an amphiphilic mode to a hydrophilic mode, in which the positively charged amino acids arginine and lysine are involved. Thus, like GroEL/GroES, the DnaK/DnaJ chaperone machine seems to perform a hydrophobic/hydrophilic flip flop, which might be of importance for its function in protein folding, protein renaturation, and insertion into membranes.

**Effect of DnaJ on the Interaction between DnaK and Hydrophobic or Amphiphilic Peptides**

— Substance P (RPKPQQFFGLM) and P4–11 (PQQFFGLM) were tested for their ability to compete for R-CMLA binding to DnaK in the absence or in the presence of DnaJ and/or ATP. In the absence of DnaJ and ATP, substance P is twice as efficient as P4–11 in competing for R-CMLA binding to DnaK (Ki = 14 and 32 mM, respectively) (Fig. 5) (in the presence of ATP, the binding of R-CMLA to DnaK was reduced, but the relative efficiencies of substance P and P4–11 in competing for R-CMLA binding to DnaK were not significantly altered (not shown)). In the presence of DnaJ and ATP, substance P becomes 25-fold more efficient than P4–11 in competing for R-CMLA binding to DnaK (Ki = 4 and 100 mM, respectively) (Fig. 5) (in the absence of ATP, DnaJ does not significantly affect the binding properties of DnaK, in accordance with the fact that ATP is required to promote an efficient DnaK-DnaJ interaction (not shown) (31)). These results support the hypothesis that DnaJ shifts the DnaK-peptide interaction from a somewhat hydrophobic mode where there is only a beneficial contribution of arginine and lysine to a more hydrophilic mode in which arginine and lysine determine the strength of the DnaK-peptide interaction.

**DISCUSSION**

In addition to hydrophobic amino acids, DnaK recognizes the positively charged amino acids arginine and lysine. One of the major properties of the chaperones BiP (11, 12, 15), DnaK (13, 16), Hsc70 (14), SecB (27), and GroEL (30, 32–33) is their ability to bind hydrophobic peptides. It enables them to interact with the hydrophobic regions that are exposed in unfolded proteins (17–20). Chaperones appear to interact also with several hydrophilic or charged amino acids; SecB, DnaK, Hsc70,
and BiP bind hydrophobic peptides with a variable preference for peptides including basic residues (12–15, 27). Previous studies and the present results suggest that three types of peptides interact with chaperones70: (i) hydrophobic peptides, (ii) amphiphilic peptides containing Arg/Lys, and (iii) Arg/Lys-rich peptides.

Hydrophobic peptides can bind to chaperones70 without requiring the presence of Arg/Lys at an extremity; most of the peptides that bind to BiP contain hydrophobic and aromatic amino acids but are not enriched in hydrophilic amino acids, or only very little (12, 15). Peptides enriched in internal hydrophobic residues are preferential DnaK substrates (13), and the remarkable specificity of DnaK for hydrophobic amino acids (16) demonstrates the importance of hydrophobic amino acid side chains for DnaK-protein interaction. In fact, the interaction of DnaK with hydrophobic sequences is probably the basis of their interaction with unfolded proteins (1, 3, 17–20).

The presence of Arg/Lys at one extremity of a hydrophobic peptide is beneficial for its interaction with DnaK. Selection of peptides by DnaK from a peptide display library showed an enrichment in hydrophobic peptides containing Arg/Lys (13). While this enrichment might reflect the influence of a neighboring negatively charged aspartate residue in the recombinant protein used for the display library, the specificity of Arg/Lys for DnaK has been confirmed by experiments with synthetic peptides (Ref. 13 and this study). The existence of an arginine/lysine binding site and its cooperation with the hydrophobic sites of DnaK supports the hypothesis that these positively charged amino acids play a role in DnaK-peptide interaction. The action of DnaK with amphiphilic peptides containing Arg/Lys at one extremity could be implicated in the membrane targeting of signal sequences (34, 35) or of membrane-spanning sequences, whose hydrophobic stretches are inserted into the membrane, while their positive N terminus remains on the cytoplasmic side of the membrane (21, 22). In protein folding and renaturation, interaction of chaperones70 with such amphiphilic bipartite sequences might allow the recognition of the interior/exterior boundary of soluble proteins, as previously suggested (13, 14). The decrease by DnaJ of the interaction between DnaK and the hydrophobic part of these sequences (this study) would permit their release for interaction between DnaK and the hydrophobic part of unfolded proteins, as previously suggested (13, 14). The decrease by DnaJ of the interaction between DnaK and the hydrophobic part of these sequences (this study) would permit their release for interaction between DnaK and the hydrophobic part of unfolded proteins, as previously suggested (13, 14).

ACKNOWLEDGMENTS—We thank Dr. A. El Yaagoubi for help during the purification of DnaK and Dr. O. Fayet (Laboratoire de Microbiologie et Génétique Moléculaire, CNRS, Toulouse, France) for the DnaK/DnaJ hyperproducing strain.

REFERENCES

1. Ellis, R. J., and Henningsson, S. M. (1989) Trends Biochem. Sci. 14, 339–342
2. Georgopoulos, C. (1992) Trends Biochem. Sci. 17, 295–296
3. Hendrick, J. P., and Hartl, F. U. (1993) Annu. Rev. Biochem. 62, 349–384
4. Liberek, K., Skowrya, D., Zylcz, M., and Georgopoulos, C. (1991) J. Biol. Chem. 266, 14491–14496
5. Liberek, K., Galitski, T. P., Zylcz, M., and Georgopoulos, C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3636–3640
6. Bukau, B., and Walker, G. C. (1989) J. Bacteriol. 171, 2337–2346
7. Wu, B., Georgopoulos, C., and Ang, D. D. (1992) J. Bacteriol. 174, 5258–5264
8. Shi, W., Zhou, J., Wild, J., Adler, J., and Gross, C. A. (1992) J. Bacteriol. 174, 6256–6263
9. Meury, J., and Kohiyama, M. (1991) J. Bacteriol. 173, 4404–4410
10. Palleros, D., Welch, W. J., and Fink, A. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5719–5723
11. Flynn, G. C., Chappell, T. G., and Rothman, J. E. (1989) Science 245, 385–390
12. Flynn, G. C., Pohl, J., Flocco, M. T., and Rothman, J. E. (1991) Nature 353, 726–730
13. Graeper, A., Zeng, L., Zhao, X., Burkholder, W., and Gottesman, M. (1994) J. Mol. Biol. 235, 848–854
14. Hightower, L. E., Sadik, S. E., and Takenaka, I. M. (1994) in The Biology of Heat Shock Proteins and Molecular Chaperones, pp. 179–207, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
15. Blond-Egliund, S., Cwirla, S. E., Dower, W. J., Lipshutz, R. J., Sprang, S. R., Sambrook, J. F., and Gething, M. J. (1993) Cell 75, 717–728
16. Richarme, G., and Kohiyama, M. (1993) J. Biol. Chem. 268, 24074–24077
17. Shrake, A., and Rupley, J. A. (1973) J. Mol. Biol. 79, 351–371
18. Miller, S., Janin, J., Lesk, A. M., and Chothia, C. (1987) J. Mol. Biol. 196, 641–656
19. Lee, B., and Richards, F. M. (1971) J. Mol. Biol. 55, 379–400
20. Vitanen, P. V., Gatenby, A. B., and Lorimer, G. H. (1992) Protein Sci., 363–369
21. Dalbey, R. E. (1990) Trends Biochem. Sci. 15, 253–257
22. Boyd, D., and Beckwith, J. (1990) Cell 62, 1031–1033
23. Zylcz, M., and Georgopoulos, C. (1984) J. Biol. Chem. 259, 8820–8825
24. Zylcz, M., Ang, D., and Georgopoulos, C. (1987) J. Biol. Chem. 262, 17437–17442
25. Zylcz, M., Yamamoto, T., McCuttrick, N., Sel, S., and Georgopoulos, C. (1985) J. Biol. Chem. 260, 759–766
26. Missiaekas, D., Georgopoulos, C., and Raina, S. (1993) J. Bacteriol. 175, 2616–2624
27. Raina, L. L. (1992) Science 257, 241–255
28. de Crom-Chanel, A., El Yaagoubi, A., Kohiyama, M., and Richarme, G. (1995) J. Biol. Chem. 270, 10571–10575
29. Liberek, C., Marsalek, J., Ang, D., Georgopoulos, C., and Zylcz, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2874–2878
30. Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K., and Hartl, F. U. (1992) Nature 356, 683–689
31. Liberek, K., Wall, D., and Georgopoulos, C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6224–6228
32. Richarme, G., and Kohiyama, M. (1994) J. Biol. Chem. 269, 7095–7098
33. Lin, Z., Schwartz, F. P., and Eisenstein, E. (1995) J. Biol. Chem. 270, 10101–10104
34. Von Heijne, G. (1986) J. Mol. Biol. 192, 287–290
35. Wild, J., Altman, E., Yura, T., and Gros, C. (1992) Genes Dev. 6, 1165–1172
36. Dingwall, C., and Laskey, R. A. (1991) Trends Biochem. Sci. 16, 478–481
37. Fenton, W. A., Kashl, Y., Furtak, K., and Horwich, A. L. (1994) Nature 371, 614–619
38. Schröder, H., Langer, T., Hartl, F. U., and Bukau, B. (1993) EMBO J. 12, 4137–4144
39. Ospikiu, J., Georgopoulos, C., and Zylcz, M. (1993) J. Biol. Chem. 268, 4821–4827
40. Ganes, J., Bujard, H., and Bukau, B. (1992) Cell 69, 833–842
41. Cyr, M. D., and Douglas, M. G. (1994) J. Biol. Chem. 269, 9788–9804
42. Wawrzynow, A., Baniecki, B., Wall, D., Liberek, K., Georgopoulos, C., and Zylcz, M. (1995) J. Biol. Chem. 270, 19307–19311
43. Jordan, R., and McMaken, R. (1995) J. Biol. Chem. 270, 4563–4569

Downloaded from http://www.jbc.org/ by guest on July 24, 2018
Specificity of DnaK for Arginine/Lysine and Effect of DnaJ on the Amino Acid Specificity of DnaK
Axelle de Crouy-Chanel, Masamichi Kohiyama and Gilbert Richarme

J. Biol. Chem. 1996, 271:15486-15490.
doi: 10.1074/jbc.271.26.15486

Access the most updated version of this article at http://www.jbc.org/content/271/26/15486

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 23 of which can be accessed free at http://www.jbc.org/content/271/26/15486.full.html#ref-list-1