Mechanism of the Targeting Action of DnaJ in the DnaK Molecular Chaperone System*

Received for publication, January 23, 2003, and in revised form, March 19, 2003
Published, JBC Papers in Press, March 24, 2003, DOI 10.1074/jbc.M300756200

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In the DnaK (Hsp70) molecular chaperone system of Escherichia coli, the substrate polypeptide is fed into the chaperone cycle by association with the fast-binding, ATP-ligated form of the DnaK. The substrate binding properties of DnaK are controlled by its two co-chaperones DnaJ (Hsp40) and GrpE. DnaJ stimulates the hydrolysis of DnaK-bound ATP, and GrpE accelerates ADP/ATP exchange. DnaJ has been described as targeting the substrate to DnaK, a concept that has remained rather obscure. Based on binding experiments with peptides and polypeptides we propose here a novel mechanism for the targeting action of DnaJ: ATP-DnaK and DnaJ and DnaK with its substrate-binding domain bind to different segments of one and the same polypeptide chain forming (ATP-DnaK), substrate-DnaJ complexes; in these ternary complexes efficient cis-interaction of the J-domain of DnaJ with DnaK is favored by their propinquity and triggers the hydrolysis of DnaK-bound ATP, converting DnaK to its ADP-ligated high affinity state and thus locking it onto the substrate polypeptide.

Hsp701 chaperones assist a variety of protein folding processes in the cell, including folding of nascent polypeptide chains, rescue of misfolded proteins, translocation of polypeptide chains through membranes, assembly and disassembly of protein complexes, and control of the biological activity of folded regulatory proteins (for a review, see Ref. 1). The chaperone action of DnaK, an Hsp70 homolog in Escherichia coli, is driven by the hydrolysis of ATP. DnaJ, an Hsp40 homolog, triggers the hydrolysis of DnaK-bound ATP and thus converts DnaK from the ATP-ligated low affinity T state to its ADP-ligated high affinity R state (2–6). The stimulation of the ATPase activity requires the conserved J-domain of DnaJ (residues 2–78 in E. coli DnaJ; Refs. 2, 4, and 7) and the adjacent G/F region (2). DnaJ itself is also capable of associating with unfolded substrates and preventing aggregation (8–11), qualifying DnaJ as a chaperone on its own. Binding of substrates requires the zinc finger-like region and the COOH-terminal region of DnaJ (12). Only full-length DnaJ, comprising both the J-domain and the substrate-binding domain, is effective, together with DnaK and GrpE, in refolding denatured firefly luciferase (12, 13). Based on these findings, it has been proposed that substrates first associate with DnaJ, which then transfers them to the substrate-binding site of Hsp70 (6, 10, 11, 14). The function of DnaJ has also been assumed to include a “catalytic activation” of DnaK for trapping target sequences (4, 15). A recent study has shown that DnaJ shares most binding motifs with DnaK, which also seems to qualify DnaJ as a targeting partner for the chaperone DnaK (16).

However, no direct experimental evidence has been reported for substrate transfer from DnaJ to DnaK, and the concept of a targeting action of DnaJ has as yet remained obscure (16). The catalytic activation of DnaK by DnaJ (4, 15) has not remained undisputed (17). Recent work (6) has shown that the stimulatory effect of DnaJ on the ATPase activity of DnaK was much stronger in the presence of a protein substrate than in the presence of a peptide substrate; however, the mechanism underlying this difference has not been clarified.

Here we present a study on the interactions of substrate with both DnaK and DnaJ. Our results show that in the case of a protein substrate, the chaperone action of DnaJ (binding to substrate) and its co-chaperone action (stimulating the ATPase activity of DnaK) co-operate in ternary (ATP-DnaK), substrate-DnaJ complexes, in which DnaJ, through a proximity effect, efficiently triggers the hydrolysis of DnaK-bound ATP and thus the conversion of DnaK from its low affinity T state to its high affinity R state that locks onto the substrate.

EXPERIMENTAL PROCEDURES

Proteins and Peptides—Nucleotide-free DnaK was purified as described (18). The protein concentration was determined photometrically with a molar absorption coefficient of ε280 = 14,500 mc2/mM (19). DnaJ and GrpE, prepared as reported (20, 21), were a gift from Dr. H.-J. Schönfeld (Hoffmann-LaRoche Basel). The concentrations of stock solutions of purified DnaK and GrpE were determined by amino acid analysis. Reduced carboxymethylated albumin (RCMLA) (bovine) was from Sigma. Peptides ala-p5 (ALLLSAARR), pA (segment of protein DnaA, 229VDALLLIDDIQFA241), and p-peptide (CALLLSAARR) were purchased with a purity >95% from Mimotopes and labeled with the environmentally sensitive fluorophor acrylodan (Molecular Probes) at the α-amino group (peptides ala-p5 and pA) or at the sulphydryl group (peptide p-peptide) and purified as described (22). For denaturing rhodanese (from Sigma), 5 mg of protein was dissolved in 1 ml of denaturing buffer (6 M guanidine HCl, 0.5 M Tris chloride, 5 mM EDTA, 1 mM dithiothreitol, pH 8.6) and incubated at room temperature for 3 h. Iodoacetamide (50 mM) was added to block the sulphydryl groups, and after 1 min at 25 °C the denatured protein was transferred to assay buffer (25 mM Hepes/ROH, 100 mM KCl, 10 mM MgCl2, pH 7.0) with a NAP-5 column (Amersham Biosciences). For digesting denatured rhodanese, a sample of the protein was incubated with immobilized TPCK-treated trypsin (Pierce) overnight at 37 °C according to the product instructions. Recombinant DnaJ1–106 was expressed and purified as described (2), and its molecular mass was confirmed by mass spectrometry. The concentrations of RCMLA, denatured rhodanese, and DnaJ1–106 were determined by the Bio-Rad protein assay.

Determination of Kinetic Constants—The binding rate constants of DnaJ for acrylodan-labeled peptides were determined as described (23). For the fast kinetic measurements, an SX18 MV stopped flow machine...
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### RESULTS AND DISCUSSION

**Peptide Binding to and Release from DnaK and DnaJ**—To establish the experimental approach for exploring the targeting action of DnaJ, the peptides to be used in this study were first tested for binding to and release from DnaK and DnaJ. Peptides, when bound to the substrate-binding domain of DnaK, stimulate the hydrolysis of DnaK-bound ATP. This increase in single-turnover ATPase activity was used as an indicator of binding of substrate to DnaK. In agreement with previous reports (16, 23), the acidic peptide pA and the D-peptide d-p5‘ did not bind to DnaK, as shown by their inability to stimulate its ATPase activity, whereas peptide ala-p5 bound to DnaK and stimulated the ATPase activity of DnaK 20-fold at saturating concentrations (Table I). The increase in fluorescence of the acrylodan-labeled peptides a-ala-p5, a-D-p5, and a-pA served for determining their rates of binding to DnaJ (Table II). DnaJ binds all three peptides, although it disfavors acidic peptides (16) and D-peptides (23).

The addition of GrpE plus ATP to a preformed ADPP• complex triggers the fast release of peptide because of the GrpE-accelerated ADP/ATP exchange in DnaK, i.e., the conversion of DnaK from the high affinity R state to the low affinity T state. The dissociation rate constant of a-ala-p5 from DnaK (k_d = 0.6 ± 0.04 s^-1) was determined by following the decrease in acrylodan fluorescence upon addition of GrpE and ATP with a stopped flow apparatus (Fig. 1). No signal change was observed after mixing GrpE plus ATP with DnaJ plus a-ala-p5, a-pA, or a-D-p5‘, indicating that GrpE plus ATP does not affect the substrate binding properties of DnaJ.

**Substrate Binding to DnaK Strictly Depends on Intrinsic Affinity**—DnaJ binds l- and D-peptides (16, 23) and exhibits a recent report with DnaK mutants, however, indicated that the intrinsic affinity of substrate to DnaK is crucial for the chaperone activity of Hsp70s (17). We used the GrpE-induced R → T conversion, which, as shown above, triggers the fast release of substrate from DnaK without affecting substrate binding to DnaJ, as a means to investigate this controversial issue. If DnaJ activated DnaK in the presence of ATP to trap a DnaK-bound peptide that DnaK cannot bind on its own (4, 15). A recent report with DnaK mutants, however, indicated that the intrinsic affinity of substrate to DnaK is crucial for the chaperone activity of Hsp70s (17). We used the GrpE-induced R → T conversion, which, as shown above, triggers the fast release of substrate from DnaK without affecting substrate binding to DnaJ, as a means to investigate this controversial issue. If DnaJ activated DnaK in the presence of ATP to trap a DnaK-bound peptide that DnaK cannot bind on its own, the transferred peptide should be released from DnaK upon the addition of GrpE. The peptides a-ala-p5 (binder of both DnaK and DnaJ) and a-pA and a-D-p5‘.

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### Table I

| Peptide ligand | k_cat |
|---------------|------|
| None          | 0.0003 ± 0.0002 |
| pA            | 0.0004 ± 0.0002 |
| d-p5‘         | 0.0004 ± 0.0002 |
| ala-p5        | 0.0006 ± 0.0001 |

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### Table II

| Rate constants of peptide binding to and release from DnaJ |
|-----------------------------------------------------------|
| Peptide ligand   | k_1 | k_2 | Kd  |
|-----------------|-----|-----|-----|
| ala-p5          | 11.800 | 0.0042 | 0.36 |
| pA              | 2000 | 0.0033 | 1.65 |
| d-p5‘           | 1700 | 0.0116 | 6.8  |

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*The rate of complex formation (k_cat) was determined with an Aminco double-beam spectrophotometer (Aminco). The rates of the first phases (amplitudes > 75% of total) were proportional to the concentration of DnaJ, and the values of k_cat and k_2 were determined from the slope and the intercept, respectively. K_cat values are calculated from k_cat and k_2 values. *k_1 and k_2 were calculated from K_cat and k_2 values taken from Ref. 23.
Targeting Action of DnaJ

DnaJ and DnaK Compete for Substrate—The co-operation between DnaJ and DnaK has been proposed to include the transfer of substrate from DnaJ to DnaK in the presence of ATP (16). This hypothesis has not as yet been experimentally verified. Here we tested an alternative, assuming that the association rate constants of DnaJ for both a-pA and a-D-p5’ were released at the same rate as in the absence of DnaK, indicating again that the release of peptide a-pA or a-D-p5’ was from DnaJ but not from DnaK (data not shown). DnaJ cannot target either the substrate to DnaK or DnaK to the substrate, i.e. DnaK selects its substrates solely on the basis of its intrinsic affinity.

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Targeting Action of DnaJ

Binding of a peptide to DnaJ does not affect the interaction between DnaJ and DnaK

| Peptide ligand | DnaJ-stimulated ATPase activity ($k_{max}$) | Rate of T $\rightarrow$ R conversion ($k_{inact}$) |
|----------------|---------------------------------------------|-----------------------------------------------|
| None           | 0.006 ± 0.002                              | 0.036 ± 0.008                                 |
| ala-p5         | 0.003 ± 0.010                              | 0.071 ± 0.010                                 |
| D-p5$^*$       | 0.005 ± 0.004                              | 0.034 ± 0.005                                 |
| pA             | 0.006 ± 0.004                              | 0.036 ± 0.005                                 |

* Measured by single-turnover ATPase assay as described (5). The concentration of peptides was 50 μM. The final concentrations of DnaK and DnaJ were 1 and 0.1 μM, respectively. For details, see “Experimental Procedures.”

b Determined by following the increases in intrinsic fluorescence of DnaK. The reaction was started by mixing (1 μM DnaK ± 20 μM peptide + 2 mM ATP) + (1 μM DnaJ + 2 μM ATP) in a stopped-flow apparatus. For details, see “Experimental Procedures.” Reported values are the means ± standard deviations of three independent experiments.

Table III

Fig. 4. Stimulation of the ATPase activity of DnaK by DnaJ and different protein and peptide ligands. The ATPase activity of DnaK was determined by single-turnover assays (for details, see “Experimental Procedures”). The final concentration of DnaK was 1 μM in all experiments. The concentrations of DnaJ and DnaJ-1-106 were 0.1 and 1 μM, respectively. dRh, denatured rhodanese; Rh fragments, the total of tryptic fragments from 20 μM denatured rhodanese. In the inhibition experiments, DnaJ was preincubated with peptides pA (100 μM) or D-p5$^*$ (100 μM) for 30 min at 25 °C before the addition of denatured rhodanese and ATP-DnaK. The data correspond to the mean values and standard deviations of three independent experiments.

DnaJ alone indicates that the binding of peptide substrate to the substrate-binding domain of DnaJ does not affect the interaction of its J-domain with DnaK, i.e. that, in the presence of a peptide, the co-chaperone action of DnaJ is independent of its chaperone action.

In the Presence of a Protein Substrate, the Co-chaperone Action of DnaJ Is Greatly Facilitated by Its Chaperone Action—A recent report has shown that DnaJ stimulates the ATPase activity of DnaK much more effectively in the presence of protein substrates, such as denatured luciferase and d$^{32}$, than in the presence of peptide (6). This result raised the question of the mechanistic basis for the higher stimulatory effect of DnaJ in the presence of protein substrates. A common feature of protein substrates is that they may form ternary complexes with DnaK and DnaJ (10, 25). The association of DnaK and DnaJ with one and the same polypeptide chain may be assumed to facilitate their interaction and thus be responsible for the high stimulatory effect of DnaJ on ATP hydrolysis by DnaK. To test this hypothesis, we used denatured rhodanese, a polypeptide substrate of 296 amino acid residues, which has been shown to form ternary complexes with DnaK and DnaJ (8).

Indeed, denatured rhodanese is more effective in stimulating the ATPase activity of DnaK in the presence of DnaJ than a peptide substrate, with 120 nM denatured rhodanese being as effective as peptide ala-p5 at a saturating concentration of 50 μM (Fig. 4). Trypsin digestion abolished the big effect of rhodanese on the stimulation of ATP hydrolysis by DnaJ. The ineffectiveness of the tryptic fragments cannot be attributed to nonbinding to DnaK, because in the absence of DnaJ, the rhodanese fragments stimulated the ATP hydrolysis to the same extent as the intact polypeptide. Apparently, trypsin does not destroy the hydrophobic regions of the polypeptide chain that are recognized by DnaK. With size exclusion chromatography, a ternary complex of denatured rhodanese polypeptide with DnaK and DnaJ was detected, whereas no complex was found with digested denatured rhodanese (Fig. 5). Apparently, the conjoint binding of DnaK and DnaJ to the same polypeptide chain greatly facilitates their interaction and underlies the high stimulatory effect of DnaJ on the ATPase activity of DnaK.
DnaK. In agreement with this notion, another permanently unfolded protein substrate, RCMLA, which binds to DnaK but not to DnaJ and thus cannot form ternary complexes with these two chaperones (8), stimulated the ATPase activity of DnaK only to the same limited extent as a peptide substrate in the presence of DnaJ (Fig. 4).

Excessive DnaJ binder peptides provide the possibility to test whether binding of DnaJ to denatured rhodanese is a prerequisite for the high stimulatory effect of denatured rhodanese. When pA or n-p5 were preincubated with DnaJ to form a peptide-DnaJ complex, the stimulatory effect of denatured rhodanese on the ATPase activity of DnaK in the presence of DnaJ decreased to the same level as in the presence of DnaJ plus peptide substrate ala-p5 (Fig. 4). Apparently, the binding of DnaJ decreased to the same level as in the presence of DnaJ plus peptide substrate ala-p5 (Fig. 4). In the presence of DnaJ1–DnaJ and a polypeptide lacking the substrate-binding domain, DnaJ1–DnaJ in stimulating the ATPase activity of DnaK (Fig. 4). It inhibits the binding of DnaJ to denatured rhodanese and thus prevents the formation of ternary complexes of denatured rhodanese with DnaK and DnaJ1. This result indicates that the efficient stimulation of the ATPase activity of DnaK by DnaJ1 in the presence of denatured rhodanese protein depends on the chaperone action of DnaJ.

To corroborate this interpretation, we examined the stimulatory effect of denatured rhodanese on the ATPase activity of DnaK in the presence of the truncated DnaJ mutant DnaJ1–106. DnaJ1–106 contains the J-domain as well as the following G/F-rich domain and is capable of accelerating the hydrolysis of DnaK-bound ATP (2). The truncated mutant lacks, however, the COOH-terminal domain that is required for substrate binding. In agreement with the previous report (2), DnaJ1–106 proved less efficient than DnaJ in stimulating the hydrolysis of DnaK-bound ATP. In the presence of denatured rhodanese, the rate of hydrolysis of DnaK-bound ATP stimulated by 1 μM DnaJ1–106 was 10 times slower than that stimulated by 0.1 μM wild-type DnaJ; in the absence of denatured rhodanese, 1 μM DnaJ1–106 turned out to be as effective as 0.1 μM DnaJ in stimulating the ATPase activity of DnaK (Fig. 4). Lacking the substrate-binding domain, DnaJ1–106 cannot form ternary complexes with ATP-DnaK and a polypeptide substrate. Indeed, in the presence of DnaJ1–106, denatured rhodanese-stimulated ATPase activity of DnaK only to the level achieved with peptide substrate ala-p5 (Fig. 4).

Conclusion—Our results show that the distribution of substrate between DnaJ and DnaK is based on direct competition rather than sequential binding and thus invalidate any mechanistic model that assumes an interaction of the same segment of the substrate first with DnaJ and then with DnaK (16). At intracellular concentrations of ATP and ADP, it will be the low affinity T state of DnaK that competes with DnaJ for the substrate (5). The stimulation of hydrolysis of DnaK-bound ATP by DnaJ is rendered highly efficient in the presence of a polypeptide substrate that provides binding sites for conjoint binding of DnaK and DnaJ, such as denatured rhodanese (Fig. 4), σ23 protein, or denatured luciferase (6). The big stimulatory effect vanishes when the formation of ternary ATP-DnaK, substrate-DnaJ complexes is not possible. In this study, formation of ternary complexes was prevented by three different experimental setups: (i) tryptic fragmentation of the substrate polypeptide, (ii) addition of exclusive DnaJ binders that specifically block the substrate-binding domain of DnaJ, and (iii) replacement of DnaJ by DnaJ1–106, which lacks the substrate-binding domain (Fig. 5).

On the basis of these results, we propose a model for the targeting action of DnaJ: ATP-DnaK and DnaJ bind to different segments of one and the same polypeptide chain to form ATP-DnaK, substrate-DnaJ complexes. The first binder to substrate can be either DnaJ (such as in the case of RepA; Refs.

TABLE IV

| Hsp40   | Source | Localization | SBD | References |
|---------|--------|--------------|-----|------------|
| DnaJ    | E. coli| N            | Yes | 12         |
| CbpA    | E. coli| N            | Yes | 36         |
| DijA    | E. coli| M            | No  | 37         |
| Sis1    | yeast  | N            | Yes | 38         |
| Ydjip   | yeast  | N            | Yes | 39         |
| Zutnin  | yeast  | N            | unknown | 40 |
| Scjip   | yeast  | N            | Yes | 41         |
| Sec63p  | yeast  | M            | No  | 42         |
| Jem1p   | yeast  | M            | unknown | 43 |
| Mdj1p   | yeast  | M            | Yes | 44         |
| Auxixin| mammalian| N             | Yes | 46         |
| Hdj1    | mammalian| N             | Yes | 47         |
| Hdj2    | mammalian| N             | Yes | 47         |

* N stands for nonmembrane protein (cytosol, lumen of endoplasmic reticulum, or matrix of mitochondria), and M stands for membrane protein.

* SBD, substrate-binding domain.

1 Mitochondria.

To corroborate this interpretation, we examined the stimulatory effect of denatured rhodanese on the ATPase activity of DnaK in the presence of the truncated DnaJ mutant DnaJ1–106. DnaJ1–106 contains the J-domain as well as the following G/F-rich domain and is capable of accelerating the hydrolysis of DnaK-bound ATP (2). The truncated mutant lacks, however, the COOH-terminal domain that is required for substrate binding. In agreement with the previous report (2), DnaJ1–106 proved less efficient than DnaJ in stimulating the hydrolysis of DnaK-bound ATP. In the presence of denatured rhodanese, the rate of hydrolysis of DnaK-bound ATP stimulated by 1 μM DnaJ1–106 was 10 times slower than that stimulated by 0.1 μM wild-type DnaJ; in the absence of denatured rhodanese, 1 μM DnaJ1–106 turned out to be as effective as 0.1 μM DnaJ in stimulating the ATPase activity of DnaK (Fig. 4). Lacking the substrate-binding domain, DnaJ1–106 cannot form ternary complexes with ATP-DnaK and a polypeptide substrate. Indeed, in the presence of DnaJ1–106, denatured rhodanese-stimulated ATPase activity of DnaK only to the level achieved with peptide substrate ala-p5 (Fig. 4).

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On the basis of these results, we propose a model for the targeting action of DnaJ: ATP-DnaK and DnaJ bind to different segments of one and the same polypeptide chain to form ATP-DnaK, substrate-DnaJ complexes. The first binder to substrate can be either DnaJ (such as in the case of RepA; Refs. 26 and 27) or ATP-DnaK, depending on the relative rate of complex formation and the binding affinities (5). The fast binding and release of substrate to and from ATP-ligated DnaK enables DnaK to scan the polypeptide chain and to bind to a favorable binding site, a segment rich in hydrophobic residues (5, 28). The location of DnaJ and DnaK in the same complex facilitates their interaction by an entropic proximity effect. Binding of DnaJ to one and the same polypeptide chain as DnaK converts the intermolecular interaction of the two proteins into an intracomplex reaction, which is faster because of the increased effective concentration of DnaJ. The intermolecular interaction is slow because the formation of the complex of the two molecules involves a considerable loss of entropy, whereas the intracomplex interaction results in little loss of entropy (29, 30). The cis-interaction of DnaJ with DnaK seems to be essential for efficient chaperone action in the refolding of denatured luciferase (31). Because the interaction of DnaJ and DnaK is transient (5, 6, 32), DnaJ may trigger spatially close DnaK molecules in succession, converting them from their ATP-ligated state to the ADP-ligated state. A single DnaJ molecule can thus trigger several DnaK molecules to lock onto and sequester aggregation-prone polypeptide segments.

The proposed model not only is fully consistent with our data but also agrees with the following previous observations: (i) DnaJ binds peptide and polypeptide substrates (16, 23, 26) and functions as a chaperone by itself (8). (ii) Efficient stimulation of the hydrolysis of DnaK-bound ATP by DnaJ is only observed in the presence of a protein substrate but not of a peptide (6). (iii) Very recently, the conjoint binding of DnaK and DnaJ to different segments of one and the same protein has been shown to be essential for the activation of latent dimeric P1 RepA into its active monomer (33). (iv) DnaKRepA and DnaJRepA, the DnaK and DnaJ homologs in Thermus thermophilus, respectively, have been found to bind to different sites of one and the same molecule of resting factor DafATth, which is supposed to be displaced by protein substrate resulting in ternary DnaK-substrate-DnaJ complexes as the active chaperone species (34). (v) Only full-length DnaJ, comprising both the J-domain and the substrate-binding domain, is active in cooperating with DnaK to assist in refolding of denatured substrates (12, 13). (vi) In cells, there is less DnaJ than DnaK (35); apparently, one DnaJ molecule has to be able to trigger several DnaK molecules. DnaJ can indeed control the chaperone cycle of DnaK at
substoichiometric concentrations (5). (vii) DnaJ and other Hsp40 homologs share a J-domain motif but vary in the other domains. A literature survey (Table IV) showed that Hsp40s either are cytosolic (or luminal or matrix) proteins with a substrate-binding domain or are membrane proteins without a substrate-binding domain that assist the translocation of polypeptide chains through membranes (5). In the latter context, the membrane, carrying the J-domain or a J-related segment, might play a role analogous to that of the substrate-binding domain located at or very close to the translocation channel through which the polypeptide chain is entering the lumen or matrix, might play a role analogous to that of the substrate-binding domain of DnaJ, i.e. to combine the J-domain, DnaK, and the polypeptide substrate into a single complex so that the propinquity effect becomes operative and rapid hydrolysis of ATP locks DnaK onto the substrate. The model may be expected to represent a mechanism underlying the chaperone function of Hsp70 systems in general.

Acknowledgments—We thank Dr. Hans-Joachim Schönfeld (Hoffmann-LaRoche, Basel, Switzerland) for valuable advice and the generous gift of DnaJ and GrpE, and Dr. Roger McMacken (Department of Biochemistry, Johns Hopkins University) for the plasmid of DnaJ–106 and its expression strain of E. coli.

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