D-Peptides as Inhibitors of the DnaK/DnaJ/GrpE Chaperone System*

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DnaK, a Hsp70 homolog of Escherichia coli, together with its co-chaperones DnaJ and GrpE protects denatured proteins from aggregation and promotes their refolding by an ATP-consuming mechanism. DnaJ not only stimulates the γ-phosphate cleavage of DnaK-bound ATP but also binds polypeptide substrates on its own. Unfolded polypeptides, such as denatured luciferase, thus form ternary complexes with DnaJ and DnaK. A previous study has shown that γ-peptides compete with γ-peptides for the same binding site in DnaJ but do not bind to DnaK (Feifel, B., Schönfeld, H.-J., and Christen, P. (1998) J. Biol. Chem. 273, 11999–12002). Here we report that γ-peptides efficiently inhibit the refolding of denatured luciferase by the DnaK/DnaJ/GrpE chaperone system (EC_{50} = 1–2 μM). The inhibition of the chaperone action is due to the binding of γ-peptide to DnaJ (K_{D} = 1–2 μM), which seems to preclude DnaJ from forming ternary (ATP-DnaK)_{m} substrate-DnaJ complexs. Apparently, simultaneous binding of DnaJ and DnaK to one and the same target polypeptide is essential for effective chaperone action.

The Hsp701 chaperone system of Escherichia coli includes DnaK and the two cohort proteins: DnaJ, a Hsp40 homolog, and GrpE. The chaperones assist protein folding by preventing and reversing off-pathway interactions that lead to aggregation (1). The key features of the Hsp70 chaperone system are the binding of unfolded hydrophobic segments of the target polypeptides to the ATP-ligated form of DnaK, the stabilization of the complex upon ATP hydrolysis, and the release of the bound ligands upon ADP/ATP exchange (1–3). This binding/release cycle is controlled by DnaJ and the nucleotide exchange factor GrpE (4, 5). DnaJ interacts with DnaK through its highly conserved NH_{2}-terminal J-domain and stimulates the hydrolysis of DnaK-bound ATP (2, 6). DnaJ also exerts a chaperone action on its own; upon association with denatured peptides, such as luciferase or rhodanese, it may prevent their aggregation (3, 6). Recently, it has been shown that γ-peptides bind to DnaJ but not to DnaK (7, 8). γ-Peptides bind to the same site of DnaJ as γ-peptides (7). Here we report that two retro-all γ-peptides derived from the NH_{2}-terminal segment of rhodanese inhibit the DnaK/DnaJ/GrpE chaperone system in refolding denatured firefly luciferase.

EXPERIMENTAL PROCEDURES

Proteins—DnaK was isolated from an overproducing strain of E. coli (JM 83) bearing the plasmid pFG9 (3). The stock solution of the protein in assay buffer (25 mM Hepes/NaOH, 100 mM KCl, pH 7.0) was stored at 80 °C and contained less than 0.1 mol of ADP/mol of DnaK (9). The concentration of DnaK was determined photometrically with ε_{590} = 14.6 mM^{-1} cm^{-1}. DnaJ and GrpE were prepared as described (10); stock solutions were stored at −80 °C in 50 mM Tris/HCl, 100 mM NaCl at pH 7.7.

Peptides—The peptide ala-p5 (ALLLSAPRR) was purchased with a purity of >90% from Chiron. The peptide was dissolved in 0.1% (v/v) acetic acid, 10% (v/v) acetonitrile and stored at −20 °C. The two γ-peptides RI1–17 (EALWKSTSVLARYLVQHV) and RI1–10 (VLARYLVQHV) were synthesized by Dr. S. Klauser in our Institute with an ABI 430 A Peptide Synthesizer (Applied Biosystems) and purified on a fast protein liquid chromatography system (Pharmacia Corp.) by gel filtration (Fractogel EMDO BIOSEC 650 S, from Merck). The molecular masses were confirmed by electrospray mass spectrometry, and the concentrations were determined by amino acid analysis. The peptides were dissolved and stored at −20 °C in 50% (v/v) acetonitrile. Control experiments excluded the possibility that the chaperone-inhibitory effect was due to contaminating acetonitrile or acetate in the refolding mixture. Both peptides were labeled with the environmentally sensitive fluorophor acrylodon (Molecular Probes) and purified as described (3). Three peaks were separated, corresponding to labeling at the α-amino group, the ε-amino group of the α-lysine residue, and double labeling at both groups. The fractions of the first peak corresponding to uniquely α-labeled RI1–17 or RI1–10 were used for the experiments.

Analytic Procedures—The ATPase activity of DnaK was determined with a single-turnover assay (5). The radioactive ADP product was separated from radioactive ATP by thin layer chromatography, and the radioactivity of ADP and ATP was determined with a liquid scintillation counter. The binding rate constants of DnaJ for acrylodan-labeled peptides were determined with a Spex Fluorolog spectrofluorimeter as described (7). The excitation wavelength was set at 370 nm (band pass, 4.6 nm) with the emission wavelength set at 520 nm (band pass, 4.6 nm). For fluorescence titration, the reactions were followed at an emission wavelength of 520 nm (band pass, 4.6 nm) until equilibrium was reached.

Preparation of Luciferase—Luciferase of Photinus pyralis was purchased from Roche Molecular Biochemicals. For denaturation, 1 mg of lyophilized material was dissolved in 1.56 ml of refolding buffer (50 mM Tris/His, 55 mM KCl, 5.5 mM dithiothreitol, pH 7.7), filtered with a 0.2-μm membrane, and precipitated by adding five volumes of acetone (−20 °C, 30 min). After centrifugation for 5 min at 10000 × g and 4 °C, the pellet was redissolved in 1.56 ml of denaturing buffer (6 μg guanidine HCl, 100 mM Tris/His, 10 mM dithiothreitol, pH 7.7) and after 0.2-μm filtration it was stored at −80 °C in 20-μl portions.

Refolding Assay—The chaperone activity of the DnaK/DnaJ/GrpE system...
\[ \text{D-Peptides inhibit the DnaK/DnaJ/GrpE Chaperone System} \]

Effect of peptides and DnaJ on ATPase activity of DnaK

ATPase activity was determined under single-turnover conditions at 25 °C as described (12). The final concentrations of DnaK and DnaJ were 1 and 0.1 \( \mu \text{M} \), respectively (for details, see “Experimental Procedures”). Luciferase was denatured in 6 M guanidine HCl as described under “Experimental Procedures”; the concentration of the stock solution was 25 \( \mu \text{M} \). The final guanidine HCl concentration (51 m\( \text{M} \)) had no effect on the ATPase activity of DnaK (not shown). In the measurements with D-peptides, DnaJ was preincubated with the D-peptides for 20 min.

**TABLE II**

| Additions         | Concentration of substrate | \( K_{\text{cat}}^\text{off} \) \( \mu \text{M} \) | \( K_{\text{off}}^\text{on} \) \( \text{s}^{-1} \) |
|-------------------|----------------------------|---------------------|---------------------|
| None              | 0.0003 \pm 0.0002          |                     |                     |
| RI1–17            | 0.0003 \pm 0.0002          |                     |                     |
| RI1–10            | 0.0004 \pm 0.0002          |                     |                     |
| DnaJ              | 0.0025 \pm 0.0020          |                     |                     |
| DnaJ + RI1–17     | 0.0054 \pm 0.0023          |                     |                     |
| DnaJ + RI1–10     | 0.0065 \pm 0.0024          |                     |                     |
| DnaJ + DnaJ       | 0.0076 \pm 0.0014          |                     |                     |
| DnaJ + DnaJ + D.luci | 0.0008 \pm 0.0002      |                     |                     |
| DnaJ + RI1–17 + D.luci | 0.0007 \pm 0.0004   |                     |                     |
| DnaJ + RI1–10 + D.luci | 0.0007 \pm 0.0002  |                     |                     |

**RESULTS AND DISCUSSION**

Binding of Peptides to DnaJ—The NH\(_2\)-terminal segment of rhodanese protein has proven to be a high affinity binder of DnaJ (11). The two D-peptides RI1–17 and RI1–10 are derived from this segment of rhodanese. To preserve the side chain topology of the rhodanese segment, both D-peptides were synthesized as retro-all-D (retro-inverso) analogs of the L-rhodanese peptide, i.e. both peptides were exclusively composed of D-amino acids, and, as compared with the reference L-peptides, possessed the reverse sequence (7).

We examined the binding of the acrylodan-labeled peptides a-RI1–17 and a-RI1–10 to DnaJ by following the change in fluorescence (3). The kinetic traces followed a double-exponential function (Fig. 1A). The rates of the first phases were found to be a linear function of the concentration of DnaJ (Fig. 1B), and the \( k_{\text{on}} \) and \( k_{\text{off}} \) values were obtained from the slope and intercept, respectively, of the plot (Table I). The rate of the second, smaller phase was independent of the concentration of DnaJ (data not shown) and thus may reflect an isomerization of the complex formed in the first phase. In accord with previous reports (7, 8), both D-peptides did not bind to DnaK, as indicated by the fact that they did not stimulate the ATPase activ-
ity of DnaK, whereas the control peptide ala-p5, a known substrate for DnaK (13), increased the ATPase activity by a factor of 20 (Table II). The peptide binding properties of DnaK are different from those of DnaJ. DnaK interacts with both the backbone and side chains of the peptides (14) and thus shows binding polarity that admits only L-peptide (7, 8). DnaJ apparently interacts exclusively with the side chains of the substrate and thus can also bind D-peptides.

Inhibition of the Chaperone Effect by D-Peptides

The refolding of denatured luciferase provides an often used in vitro assay for the chaperone action of the DnaK/DnaJ/GrpE system (15). Previous studies have shown that for efficient refolding the presence of all three chaperones is necessary (15) and that denatured luciferase forms ternary complexes with DnaK and DnaJ (6). However, it has remained unclear whether the joint binding of DnaK and DnaJ to different sites of the same target polypeptide chain is prerequisite for the chaperone action. The finding that D-peptides bind exclusively to DnaJ and compete with L-peptide ligands for the same binding site (7, 8) provides an approach to explore this question.

We tested the effect of the D-peptides on the refolding of denatured luciferase in the presence of DnaK, DnaJ, GrpE, and ATP. Upon preincubation of DnaJ with RI1–17 or RI1–10, the yield of refolding decreased with increasing concentration of the D-peptide (Fig. 2A). Both RI1–17 and RI1–10 were effective in inhibiting the chaperone-assisted refolding of luciferase, with EC50 values of ~2 μM (Fig. 2B) and ~1 μM (Fig. 2C), respectively. The EC50 values correlated with their binding affinities for DnaJ (Table I). In a control experiment, the addition of either peptide alone to denatured luciferase did not affect its spontaneous refolding (data not shown). Because both D-peptides bind exclusively to DnaJ and compete with L-peptide ligands for the same binding site (7), the observed inhibition of DnaK, whereas the control peptide ala-p5, a known substrate for DnaK (13), increased the ATPase activity by a factor of 20 (Table II). The peptide binding properties of DnaK are different from those of DnaJ. DnaK interacts with both the backbone and side chains of the peptides (14) and thus shows binding polarity that admits only L-peptide (7, 8). DnaJ apparently interacts exclusively with the side chains of the substrate and thus can also bind D-peptides.

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DnaJ does not affect the interaction of the J-domain of DnaJ 10-fold (Table II). The binding of peptides RI1–H18528 stimulated the ATPase activity of DnaK only peptide complexes in stimulating the ATPase activity of DnaK (DnaK) and to form ternary (ATP-DnaK) substrate-DnaJ complexes. In the presence of denatured luciferase, DnaJ stimulated the ATPase activity of DnaK—100-fold (Table II and Ref. 17), whereas preformed DnaJ-R11–17 or DnaJ/R11–10 stimulated the ATPase activity of DnaK only 10-fold (Table II). The binding of peptides R11–17 or R11–10 to DnaJ does not affect the interaction of the J-domain of DnaJ with the ATPase domain of DnaK; the stimulatory effect of DnaJ on the ATPase activity of DnaK in the presence of either D-peptide was the same as that in the absence of peptide inhibitor (Table II). Apparently, the ineffectiveness of DnaJ-D-peptide complexes in stimulating the ATPase activity of DnaK in the presence of denatured luciferase is due to the incapability of the DnaJ-D-peptide complexes to bind to denatured luciferase. These results corroborate the notion that D-peptides inhibit the chaperone action of the DnaK/DnaJ/GrpE system by precluding DnaJ from engaging in ternary (ATP-DnaK) substrate-DnaJ complexes.

Conceivably, the conjoint binding of DnaJ and DnaK to one and the same polypeptide chain facilitates the interaction of the J-domain of DnaJ and the ATPase domain of DnaK by a propinquity effect (16). The cis-interaction of DnaJ with DnaK seems not only to be necessary for efficient chaperone-mediated refolding of target proteins but has also been reported to be required for the activation of latent P1 RepA dimer into its active monomer (18). Why is the binding of DnaK and DnaJ to one and the same unfolded target polypeptide necessary for the chaperone action of the DnaK system? Rapid triggering of the hydrolysis of DnaK-bound ATP might be essential for feeding the substrate protein into the chaperone cycle. Hydrolysis of ATP converts DnaK from its low affinity state to its high affinity ADP-ligated state. Without the cis-action of DnaJ, the substrate would cycle on and off ATP-DnaK, without chaperone action being effected.

Concluding Remarks—The exclusive binding of D-peptides to the substrate-binding site of DnaJ (7, 8) specifically allows inhibition of the association of DnaJ with a substrate polypeptide. Substrate-induced effects in the DnaK/DnaJ/GrpE system that are due to the binding of substrate to DnaJ may thus be discriminated from effects that are due to the binding of substrate to DnaK. Using this approach, we provided evidence that the chaperone action of the DnaK/DnaJ/GrpE system depends on the formation of ternary (ATP-DnaK), substrate-DnaJ, complexes. The acceleration of the chaperone cycle consequent to the formation of ternary complexes, perhaps together with an as yet unknown topological or conformational constraint inherent in the cis-interaction of DnaK and DnaJ, appears to be a prerequisite for efficient chaperone action.

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