d-Peptide Ligands for the Co-chaperone DnaJ*

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The molecular chaperone DnaK, the Hsp70 homolog of Escherichia coli, binds hydrophobic polypeptide segments in extended conformation. The co-chaperone DnaJ (Hsp40) has been reported to bind native and denatured proteins as well as peptides. We tested pseudo-peptides of d-amino acids as ligands for both chaperones. In comparison to the parent all-l peptide, these mimetics had either enantiomorphic side chain positions combined with retained main chain direction (normal all-d peptide) or unchanged side chain topology together with reverse direction of the peptide backbone (retro all-d peptide). The peptides were labeled with acrylodan (α), and their binding to DnaK and DnaJ was monitored by the accompanying increase in fluorescence intensity. The parent all-l peptide a-CALLLA-SAARR bound to both DnaK (K_d = 0.1 μM) and DnaJ (K_d = 9.2 μM). In contrast, the normal all-d and retro all-d peptides did not bind to DnaK; they bound, however, to DnaJ with K_d values of 6.8 μM and 0.9 μM, respectively. The emission spectra of the DnaJ-bound peptides suggests that DnaJ bound both d-peptides with the same main chain direction as l-peptides. Binding of the normal all-d and all-l peptides inhibited the DnaJ-induced stimulation of DnaK ATPase. However, binding of the retro all-d analog to DnaJ did not impair the stimulation, indicating the existence of separate binding sites for peptides and DnaK.

Chaperones of the Hsp701 family fulfill essential functions in protein folding by preventing and reversing off-pathway interactions that lead to aggregation (1, 2). Hsp70s are also required for membrane translocation of precursor polypeptides (3, 4) and participate in the degradation of misfolded proteins (2).

The Hsp70 chaperone system of Escherichia coli comprises the Hsp70 homolog DnaK and the co-chaperones DnaJ and GrpE. DnaK consists of a 44-kDa ATPase domain, the crystal structure of which has recently been determined in a complex with a dimer of truncated GrpE (5), and a 27-kDa peptide-binding domain, the crystal structure of which has been solved in a complex with a synthetic peptide ligand (6). The chaperone effects of the DnaK/DnaJ/GrpE system are based on the ability of DnaK to bind extended hydrophobic segments of proteins in a reversible manner and possibly to exert conformational work on them (2, 7). Binding and release of polypeptides are modulated by ATP binding and hydrolysis (8), which on their part are controlled by the co-chaperones GrpE and DnaJ, respectively (7, 9). DnaJ interacts with DnaK through its highly conserved N-terminal J-domain (10, 11). However, DnaJ appears to exert also a chaperone function of its own (12). The co-chaperone associates with denatured polypeptides independently of DnaK, e.g. luciferase and rhodanese (13, 14), preventing their aggregation and targeting them to DnaK.

In previous studies, we have found various peptides derived from the presequence of mitochondrial aspartate aminotransferase to be high affinity ligands of DnaK (7). In a search for peptide mimetics that might be used as inhibitors of the chaperone system, we tested normal all-d and retro all-d (retro-inverso) analogs of a parent all-l peptide for binding to DnaK and DnaJ. In the normal all-d peptide mimetics, all-d-amino acid residues have been replaced by the corresponding L-enantiomers; the main chains of these peptide analogs have the same direction as in the parent peptides, whereas the side chains occupy enantiomorphic positions. In contrast, retro all-d peptides, i.e. mimic peptides of d-amino acids with, as compared with the reference l-peptides, reverse sequence, exhibit in extended conformation the same side chain topology as their native counterparts (15). In certain cases they may therefore retain the biological activity of the reference l-peptides while being much less susceptible to proteolytic degradation (16–18).

Here we show that the co-chaperone DnaJ binds the tested pseudo-peptides with similar affinities as the parent all-l peptide. In contrast, DnaK did not bind any of the d-peptides with measurable affinity.

EXPERIMENTAL PROCEDURES

Purification of Proteins—DnaK from an overproducing strain of E. coli bearing the plasmid pTTQ19dnak* was purified and prepared for experimentation as described previously (19). Purified DnaK contained less than 0.1 mol of ADP/mol of DnaK (20). Protein concentration was determined photometrically with ε_280 = 14.6 mM⁻¹ cm⁻¹ (20). DnaJ and GrpE were prepared as described (21, 22); stock solutions were 120 μM and 240 μM, respectively, in 50 μM Tris/HCl, 100 mM NaCl, pH 7.7. Proteins were stored at −80 °C.

Preparation of Peptides—The all-l peptide p5′ (NH₂-CALLLA-SAARR-COOH), normal all-d peptide p5′ (NH₂-callasarr-COHN), two retro all-d analogs of p5′ (retro all-d p5′: NH₂-craasllae-COHN and retro all-d p5′: NH₂-craasilla-COHN), and the N-terminal rhodanese peptide (NH₂-VHQLYRLAStVKLAE-COOH) were purchased with a purity of >90% from ANAWA (all sequences are indicated in the conventional CO-NH direction of peptide bonds). Peptides were labeled at their sulfhydryl groups with acrylodan (from Molecular Probes, Eugene, OR) and purified as published (19). The mass of monolabeled peptides was checked by mass spectrometry. Labeled and unlabeled peptides were stored at −20 °C as 1 mM stock solutions in 0.1% acetic acid containing 10 mM dithiothreitol. Samples of all-l peptide p6 (NH₂-CARSLLLSS-COOH), synthesized and purified as described (19), and normal all-d p5′ were labeled at the sulfhydryl group with N-ethyl-2-

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‡The abbreviations used are: Hsp70, 70-kDa heat shock protein; NEM, N-ethylmaleimide; *NEM, N-l-ethyl-2-3H]maleimide; α-p5′, acrylodan-labeled peptide p5′.

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3Hmaleide (‘NEM) as follows. 50 µl of ‘NEM (50 µCi, 60 Ci/mmol) in pentane was mixed with the same volume of 50 mM HEPES, pH 7.0. Pentane was removed by evaporation, and 5 µl of a 1 mM peptide solution were added. After 5 min, 50 µl of 5.3 mM nonradioactive NEM was added and the solution was kept for 1 h at 25 °C. ‘NEM-labeled peptides were purified to homogeneity by high performance liquid chromatography using an analytical reversed-phase C-8 column. The specific radioactivity of the peptides was about 7000 cpm/pmol.

Fluorescence Measurements—Fluorescence spectra of the acrylodan-labeled peptides and their reactions with the chaperones were recorded with a Spex Fluorolog spectrofluorimeter. The excitation wavelength was set at 370 nm (band pass 4.6 nm). For fluorescence titration, the labeled peptides and their reactions with the chaperones were recorded for 30 min at 25 °C. Differences in fluorescence at 520 nm were entered into the equation.

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Size Exclusion Chromatography—Gel filtration experiments were performed on a fast protein liquid chromatography system from Amersham Pharmacia Biotech using a Superose 12 column (10 × 300 mm) at a flow rate of 0.5 ml/min. Running buffer was 25 mM Tris/HCl, 100 mM KCl, pH 7.5. The peptides were detected by their absorption at 225 nm. The calculated value of Kd was 0.9 µM.

RESULTS AND DISCUSSION

Binding of Acrylodan-labeled B-Peptides to DnaJ—The labeled peptide a-p5 (CLALLSAARR), which represents part of the presequence of mitochondrial aspartic aminotransferase, has previously been shown to bind both to DnaK and DnaJ, with Kd values of 0.06 µM and 37 µM, respectively (7). The similar all-1 peptide a-p5’ (CALLLSAARR), which binds to DnaK with a Kd of 0.1 µM (not used), was used as reference peptide in this study. Addition of DnaJ to normal all-b a-p5’ and retro all-b a-p5’ (for the structures of the peptides, see Table I) resulted in an increase in fluorescence and a shift in the emission maximum from 526 nm to 520 nm, indicating binding of these peptides (Fig. 1). Similar binding-induced changes in fluorescence were observed with all-l a-p5 and all-l a-p5’ (Table I), suggesting that the acrylodan-labels of DnaJ-bound normal all-b a-p5’ and retro all-b a-p5’ are located in an environment similar to that of the DnaJ-bound all-l peptides. In contrast, addition of DnaJ to retro all-b p5’-a, which has the fluorophore attached to its C-terminus, shifted the fluorescence maximum to 490 nm. This more pronounced blue shift (Fig. 2) is an exception among all peptides tested and suggests that the acrylodan label of retro all-b p5’-a is situated at the opposite site of the peptide-binding region of DnaJ (Table I). This interpretation implies that retro all-b p5’-a, as the other tested B-peptides, binds with the same peptide backbone direction as the I-peptides. All-l and normal all-b peptides were bound in a biphasic process, while the binding of both retro all-b peptides could be fitted to single-exponential functions (Fig. 1). The two retro all-b peptides are the most strongly binding ligands; normal all-b a-p5’ binds with lower affinity, similar to that of all-l a-p5’ (Table I). For comparison, the 17-residue N-terminal peptide of rhodanese has been reported to bind with a Kd < 20 nm to DnaJ but not to DnaK (23). We used this high affinity peptide ligand to check the specificity of binding of the tested B-peptides. Addition of 4 µM unlabeled rhodanese peptide to preformed complexes of normal all-b a-p5’ and retro all-b p5’-a with DnaJ (conditions as described in the legend to Fig. 1, except that 1.5 µM DnaJ was used) decreased the fluorescence signal of the DnaJ-bound labeled peptides in a time-dependent manner.
**TABLE II**

*Stimulation of DnaK ATPase by DnaJ in the presence and absence of peptides*

| Peptide                | Concentration | DnaJ | $k_{cat}$ | μM | min⁻¹ |
|------------------------|---------------|------|-----------|----|-------|
| None                   | –             | –    | 0.03      |    |       |
| Retro all-L p5’        | 50            | –    | 0.04      |    |       |
| Normal all-L p5’       | 50            | –    | 0.04      |    |       |
| Normal all-L p5’       | 50            | –    | 0.11      |    |       |
| None                   | +             | 1.9  | 1.9       |    |       |
| Retro all-L p5’        | 50            | +    | 2.6       |    |       |
| Retro all-L p5’        | 150           | +    | 0.82      |    |       |
| Normal all-L p5’       | 150           | +    | 0.23      |    |       |
| Normal all-L p5’       | 150           | +    | 0.23      |    |       |

**Inhibition of the Stimulatory Effect of DnaJ on DnaK ATPase Activity**—The rate of steady-state ATP hydrolysis of DnaK is stimulated by DnaJ up to 200-fold (2, 7). Because the D-peptides bind to DnaJ but not to DnaK, we investigated their effect on the ability of DnaJ to stimulate the steady-state ATPase activity of DnaK (Table II). We preincubated DnaJ with saturating concentrations of peptides, then added DnaK plus GrpE and followed the generation of ADP. Peptide retro all-L p5’ slightly stimulated the ATPase activity in the presence of DnaJ. The binding of retro all-L p5’ without concomitant inhibition of DnaK ATPase indicates that the co-chaperone DnaJ possesses two separate binding sites for peptides and DnaK. Normal all-L p5’ and all-L p5’ markedly decreased the DnaJ-stimulated ATPase activity of DnaK to 44% and 12%, respectively, of the activity in the absence of peptide (Table II). It remains to be explored whether this inhibitory effect is due to direct binding of these peptides to the DnaJ recognition site of DnaJ or to an allosteric effect caused by binding to the peptide-binding site that hinders or prevents interaction with DnaJ.

**Implications**—In contrast to DnaK, DnaJ recognizes peptide-mimetics that are composed exclusively of D-amino acids. For DnaK, neither the identical positions of the side chains in the case of retro all-L analogs nor the same direction of the peptide backbone (normal all-L peptide) suffice to mimic the reference peptide p5’. Interactions with both the peptide back-
bone and the side chains seem to limit the binding capacity strictly to L-peptides. DnaJ, however, seems to be less exacting with respect to the spatial orientation of side chains of the target peptide. All tested L- and D-peptides were recognized as ligands, binding with different spectral and kinetic features. 2) Spectral differences between DnaJ complexes of peptide retro all-D p5'-a that has the fluorophore attached to the C-term and the other peptides (Table I) suggest that retro all-D p5'-a binds with unchanged backbone direction and thus, like normal all-D a-p5', with enantiomorphic side chain positions. 3) The peptides all-L a-p5', normal all-D a-p5', and retro all-D p5'-a differentially inhibit DnaJ in stimulating the DnaK ATPase activity, i.e. the extent of DnaJ-induced ATPase activity of DnaK depends on the nature of the DnaJ-bound peptide ligand. 4) DnaJ apparently possesses two binding sites, one for polypeptides, binding all-L as well as all-D peptides, and one for interaction with DnaK. Upon binding of peptides, DnaJ may control the action of the DnaK system by means of an allosteric regulation of its DnaK-interaction site.

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