Multiple Molecules of Hsc70 and a Dimer of DjA1 Independently Bind to an Unfolded Protein*•§

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Protein folding is a prominent chaperone function of the Hsp70 system. Refolding of an unfolded protein is efficiently mediated by the Hsc70 system with either type 1 DnaJ protein, DjA1 or DjA2, and a nucleotide exchange factor. A surface plasmon resonance technique was applied to investigate substrate recognition by the Hsc70 system and demonstrated that multiple Hsc70 proteins and a dimer of DjA1 initially bind independently to an unfolded protein. The association rate of the Hsc70 was faster than that of DjA1 under folding-compatible conditions. The Hsc70 binding involved a conformational change, whereas the DjA1 binding was bivalent and substoichiometric. Consistently, we found that the bound 14C-labeled Hsc70 to the unfolded protein became more resistant to tryptic digestion. The gel filtration and cross-linking experiments revealed the predominant presence of the DjA1 dimer. Furthermore, the Hsc70 and DjA1 bound to distinct sets of peptide array sequences. All of these findings argue against the widely proposed hypothesis that the DnaJ-bound substrate is targeted and transferred to Hsp70. Instead, these results suggest the importance of the bivalent binding of DjA1 dimer that limits unfavorable transitions of substrate conformations in protein folding.

Molecular chaperones are a group of key molecules that act in numerous biological processes by assisting in conformational transitions of various proteins within the cell. The 70-kDa heat shock cognate protein (Hsc70, also known as Hsp73) in the cytosol engages in the folding of unfolded or newly translated proteins, prevention of misfolded proteins to form aggregates, assembly of protein complexes for signal transduction, translocation of proteins across organelle membranes, and degradation of proteins (reviewed in Refs. 1–3). Protein folding is the prominent function.

The Hsp70 protein usually functions as a chaperone system with various combinations of components including an Hsp70 protein, a DnaJ/Hsp40 protein, and a cochaperone(s). Several cytosolic DnaJ proteins have been investigated, and the biological significance of the canonical DnaJ proteins DjA1 (DnajA1: dj2/HSDJ/hdj2) and DjA2 (DnajA2: dj3/HIRIP4/hdj3) have been reported (4–6). The canonical DnaJ proteins are represented by Escherichia coli DnaJ and classified as type 1 (7). The general features of the Hsp70 reaction cycle were mainly obtained from studies of the bacterial Hsp70 (DnaK) system (reviewed in Refs. 8 and 9). The chaperone activity of the Hsp70 is regulated by the nucleotide state. Hsp70-ATP induces an “open” state of the adjacent substrate-binding domain, and this state allows for the fast exchange of substrate polypeptides. Hsp70-ADP induces a “closed” state that tightly binds a substrate (slow exchange). These two states cycle with a rate determined primarily by the Hsp70 ATPase and nucleotide exchange. The cycle is further regulated by other components: a DnaJ/Hsp40 protein accelerates the ATPase of the Hsp70 (10), and the ADP/ATP exchange rate is enhanced by a group of chaperones called nucleotide exchange factors (11). Many species of DnaJ/Hsp40 proteins and chaperones are present in the cytosol. Some have a defined role in a specific biological process, whereas the others have redundant or alternative roles. How the individual components discriminate the chaperoning substrate proteins remains to be elucidated. The direct measurement of the initial binding processes of the components is obviously lacking.

The binding motifs for bacterial Hsp70 component (12, 13) and eukaryotic DnaJ proteins (14) have hydrophobic characteristics. These binding motifs are frequently present in protein sequences and are usually buried in the native structure (12). Exposure of these hydrophobic regions in an unfolded state frequently results in irreversible misfolding and aggregate formation. Most of the direct binding studies have investigated chaperone interactions with peptide substrates or aggregate-free proteins because of various technical difficulties in handling an unfolded protein.

Many studies have focused on the folding mechanism of the Hsp70 system (8, 9). The most comprehensive model shows that DnaJ binds a substrate polypeptide and then transfers the bound substrate to the substrate-binding cavity of Hsp70. This model couples the substrate transfer and ATP hydrolysis and provides an elegant explanation for the cooperative and sequential interactions among the substrate and the chaperones (15). The substrate transfer model for DnaJ is consistent with the published findings regarding several DnaJ proteins (16). However, a precise analysis is still needed to confirm the generality of the model by measuring the kinetics and stoichiometry of the chaperone binding to a whole molecule of substrate protein.

The current study was conducted to further elucidate the folding mechanism of the Hsc70 chaperone system. A folding-compatible concentration of individual components to refold

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An unfolded protein was examined. A surface plasmon resonance (SPR) method was developed to monitor the initial process of chaperone binding to the unfolded protein. A conformational change of the bound Hsc70 to the unfolded protein was suggested by tryptic digestion. The predominant presence of DjA1 dimer was detected by gel filtration and cross-linking experiments. The binding sites for the chaperones were investigated by screening 180 peptides derived from the substrate protein. All of the results clearly delineated the unexpected and essential features of the substrate recognition process by the Hsc70 and the DjA1 and argue against the generality of the hypothesis for the DnaJ proteins.

**EXPERIMENTAL PROCEDURES**

**Screening of Cellulose-bound Peptides**—A peptide library covering the entire firefly luciferase was prepared by automated spot synthesis (PepSpot peptides; JPT Peptide Technologies GmbH). Approximately 5 nmol of peptides were synthesized in each grid with a spacing of 0.37 cm. The peptides were C-terminally attached to cellulose membrane via a (β-Ala)₂ spacer. The screening followed previous procedures (12, 13) with some modifications. Rat Hsc70 (500 nM, purified as described in Ref. 4) was allowed to react with the PepSpot peptides as described for DnaK by Rüdiger et al. (12). Unbound Hsc70 was extensively washed out with Tris-buffered saline buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl) containing 0.05% Tween 20, and tightly bound Hsc70 on the PepSpot peptides was probed with Hsc70 antibody (B6; Stressgen). Human H₁,DjA1 (100 nM, purified as described in Ref. 4) was allowed to react with the PepSpot peptides as described for DnaJ (13). Unbound DjA1 was removed with Tris-buffered saline, and peptide-bound DjA1 was electrotransferred onto membranes. Transferred DjA1 was probed with a monoclonal antibody (MS225; NeoMarkers). The chaperone-antibody complexes on membranes were detected with appropriate peroxidase-conjugated secondary antibodies and enhanced chemiluminescence kits (GE Healthcare) using a chemiluminescence imaging system (LAS4000; Fujifilm Corp.).

**Preparation of Liver Extract**—Freshly dissected mouse liver was washed twice and homogenized with three strokes of Teflon pestle in 2 volumes of homogenization buffer (10 mM Hepes-NaOH, pH 7.2, 250 mM sorbitol, 50 mM KCl, 1 mM EGTA, 1 mM NaF, 0.1 mM Na₂VO₄, and protease inhibitors (Roche Applied Science)) at 4 °C. To obtain the cytosolic extract, the homogenate was sequentially centrifuged at 1,000 × g for 10 min, 10,000 × g for 10 min, and 100,000 × g for 30 min. Typically, the protein concentration of the extracts ranged from 25 to 30 mg/ml. The extract was divided into aliquots and stored at −80 °C until use. The luciferase refolding assay was done as described (5).

**SPR Analysis**—The SPR measurement was conducted with a BIAcore 2000 system (GE Healthcare) at 25 °C in HKMB buffer (20 mM Hepes-KOH, pH 7.4, 120 mM potassium acetate, 1.6 mM magnesium acetate, 1 mg/ml bovine serum albumin) at a flow rate of 20 µl/min. The chaperone solutions were assembled on ice 3 min before the injection. Firefly luciferase (Sigma) was prepared at 2–10 µg/ml in 10 mM sodium acetate (pH 4.0) and coupled to the CM5 sensor chip via the standard amine coupling procedure. The coupled protein was completely denatured with multiple injections of 6 M guanidine HCl. To unfold the denatured protein in a chaperone-accessible conformation, 5 µl of 50 mM NaOH was passed through before sample injection. Similar binding was obtained with unfolding by guanidine HCl. After washing with HKMB buffer for 2 min, a chaperone protein was allowed to bind for 3 min. The sensor chip was regenerated with 15 µl of NaOH. The regeneration was conducted initially with Bag1 (5) and ATP when Hsc70 protein was injected and subsequently with NaOH. After 10 cycles of binding and regeneration, the decrease of response was <10% for the Hsc70 and <4.0% for the DjA1. The mass of the coupled protein was estimated from the resonance units according to the following equation: 1 resonance unit = 1 pg/mm² (17).

**Kinetic Analysis of Sensorgram Data**—The kinetic constants were calculated from the sensorgrams using the BIAevaluation software (version 4.1) with predefined models. A molecular mass of 70.9 kDa was used for Hsc70 monomer and 91.7 kDa for DjA1 dimer to obtain the kinetic constants. The response was collected every 1 s, and all binding curves were subtracted by a mock reference flow cell. The data points corresponding to 20 s after the injection start and 20 s after the injection end were excluded from the fitting procedure as recommended in the BIAevaluation software handbook. The goodness of fit was assessed by inspecting the statistical value (χ² < 2) and the residuals.

**Tryptic Digestion of Hsc70**—The Hsc70 protein was ¹⁴C-labeled by reductive methylation using [¹⁴C]formaldehyde (PerkinElmer; 2.1 GBq/mmol) and NaBH₄ (18). Luciferase was coupled to the CM-Sepharose beads as described to the sensor chip. The coupled luciferase (15 µl of a packed slurry of beads, 1.4 µg of luciferase/µl of beads) was successively unfolded with guanidine HCl and NaOH and rapidly exchanged to HKMB buffer using the Ultrafree-MC centrifugal filter device (Millipore; 0.1-µm pore). The unfolded luciferase was incubated with 20 µl of the HKM buffer containing 4 µg of the ¹⁴C-labeled Hsc70 (25 KBq) and 0.1 mM ATP. After 3 or 40 min of incubation, the solution was spun down, and the remaining protein in the filter cup was treated with 12 ng of trypsin in 20 µl of the buffer for 5 min. The treatment was halted by the addition of 3 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and the filtrate was recovered by centrifugation. The remaining proteins in the filter cup was further extracted with the sample buffer containing 4% SDS, and the filtrates were combined for analysis. The ¹⁴C-labeled Hsc70 (2 µg) in a nucleotide-bound state was also tested for the trypctic digestion. The samples were separated by SDS-PAGE and visualized by fluorography.

**Detection of DjA1 Dimer**—The DjA1 protein at 1 mg/ml was cross-linked with 4 mM of dimethyl suberimidate in 0.2 M triethanolamine HCl buffer (pH 8.5). The excess cross-linker was inactivated with 0.2 M ammonium acetate. The sample was analyzed by SDS-PAGE according to the procedure of Weber and Osborn (19). DjA1 protein (150 µg) was separated by a G3000SwXL gel filtration column (Tosoh Corp.) using a Hitachi 655 high pressure liquid chromatography system at a flow rate of 1.0 ml/min. The running buffer was 50 mM sodium phosphate buffer (pH 7.4) and 0.5 mM NaCl, and absorbance at 280 nm
Hsc70 and DjA1 Binding to an Unfolded Luciferase—Various amounts of luciferase was coupled covalently to sensor chips and unfolded by a denaturant completely, and individual chaperone binding was investigated (Fig. 2, A and B). The amount of chaperone binding increased almost linearly with the amount of coupled protein (Fig. 2C). The binding of Hsc70 at the end of the association period well exceeded to the amount of coupled luciferase (the molar ratio was 2.4). Surprisingly, the amount of bound DjA1 was much lower than that of the coupled protein (the molar ratio was 0.18, DjA1 as a dimer). Although these molar ratios merely represented the amounts of chaperone binding during the course of substrate recognition, a single molecule of unfolded luciferase could bind several Hsc70 proteins and a substoichiometric amount of DjA1 dimer.

As expected, the binding of Hsc70 was ATP-dependent, whereas that of DjA1 was ATP-independent (supplemental Fig. S2). Neither Bag1 nor glutathione S-transferase (a control protein) bound to the coupled protein.

Unfolded Protein Dynamically Changes Its Conformation—The chaperone injection was started just after the unfolding of coupled protein at the indicated times (Fig. 2, D and E). The binding of the individual chaperone was apparently decreased in a first order reaction (Fig. 2F). The unfolded luciferase lost its binding capacity for DjA1 more rapidly (t_1/2 = 13 min) than that for Hsc70 (t_1/2 = 63 min). The binding capacities for Hsc70 and for DjA1 were estimated to be 95 and 80%, respectively, when unfolded protein was left for 2 min before chaperone injection. These results demonstrate that the unfolded and binding-competent protein on a chip gradually hides its binding sites for the chaperones by an intramolecular conformational change. However, the decrease of the binding capacity (R_{max}) was relatively small within the time necessary for monitoring the association. We then attempted to evaluate the chaperone binding.

Nature of Luciferase Refolding—Both rabbit reticulocyte lysate and the reconstituted Hsc70 system have a potent chaperone activity in refolding denatured luciferase (4, 5). As shown in Fig. 1A, an extract from the mouse liver could efficiently refold denatured luciferase at 10 mg/ml. The concentrations of the purified Hsc70 and the DjA2 (as well as DjA1) used for the reconstitution were set to similar levels in comparison with those in the extract at 10 mg/ml (supplemental Fig. S1). There are three kinds of nucleotide exchange factors for the Hsc70 system (11), and Bag1 was selected for the experiments. A series of experiments with decreasing amounts of individual components showed that a substoichiometric amount of DjA2 but not Hsc70 could achieve significant refolding (Fig. 1B). DjA1 worked a similar manner as well (4). The absence of Bag1 resulted in slight reduction in total yield of refolding after 60 min, but the refolding proceeded almost linearly with a lag time at the initial 10 min as reported earlier (5). Next, the Hsc70 system was added with staggered timing to assess the folding competence of the unfolded protein (Fig. 1C). The unfolded luciferase lost its folding competence rapidly (t_1/2 < 3.5 min) in the first few minutes. The loss of competence was slower in the subsequent period (t_1/2 = 13 min). Approximately 60% of the competence was retained after 8 min of incubation without chaperones. These results prompted an investigation of the chaperone interaction with the unfolded protein using the SPR technique.

RESULTS

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Several Hsc70 Proteins and Substoichiometric Numbers of DjA1 Bind to an Unfolded Luciferase—Various amounts of luciferase was coupled covalently to sensor chips and unfolded by a denaturant completely, and individual chaperone binding was investigated (Fig. 2, A and B). The amount of chaperone binding increased almost linearly with the amount of coupled protein (Fig. 2C). The binding of Hsc70 at the end of the association period well exceeded to the amount of coupled luciferase (the molar ratio was 2.4). Surprisingly, the amount of bound DjA1 was much lower than that of the coupled protein (the molar ratio was 0.18, DjA1 as a dimer). Although these molar ratios merely represented the amounts of chaperone binding during the course of substrate recognition, a single molecule of unfolded luciferase could bind several Hsc70 proteins and a substoichiometric amount of DjA1 dimer.

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Hsc70 Binding Involves a Conformational Change, whereas DjA1 Binds at Two Sites—Sensorgrams were obtained with various concentrations of individual chaperones in the presence of ATP throughout (Fig. 3, A and B). The slopes of the secondary plots were not linear (supplemental Fig. S3), indicating that the binding process could not be described by a single reaction. Initial attempts to fit the experimental data globally to the various predefined models yielded unsatisfactory results (poor r^2 values; data not shown). This may be due to the metastable nature of unfolded proteins (Fig. 2, D–F). The binding competence for the chaperones was decreased in a time-dependent manner (and chaperone concentration-dependent), and this severely affected the association rate constants and the maxi-
**Hsc70 and DjA1 Binding to an Unfolded Protein**

**FIGURE 2. Binding of chaperones to the coupled and unfolded luciferase.** A and B, binding of Hsc70 (200 μg/ml or 2.8 μM) and DjA1 (20 μg/ml or 0.22 μM as a dimer) to various amounts of coupled luciferase. C, the molar ratios for binding of chaperones to the luciferase on sensor chips. The bound Hsc70 (A) and DjA1 (B) at the ends of association phases were plotted against amount of the luciferase. D and E, sensorgrams for delayed injections of Hsc70 (80 μg/ml) and DjA1 (20 μg/ml). The luciferase on the chip was unfolded prior to chaperone injection (arrow) and washed by the running buffer for the indicated times. F, decrease of chaperone binding capacity. The bound chaperones at the ends of association phases (D and E) were plotted versus the delayed injection times.

Hsc70 and DjA1 binding to an unfolded protein. The ability of unfolded luciferase on a chip to be folded by the individual addition of Hsc70 and DjA1 at folding-compatible concentrations was investigated. As illustrated in Fig. 4, a DnaJ protein binds to an Hsp70-ATP in solution with a dissociation constant of 0.5–0.6 μM (20, 21), thus leading to the rapid formation of Hsp70-ADP. Therefore, the simultaneous addition of Hsc70 and DnaJ protein lowers the concentrations of both Hsc70-ATP and free DnaJ protein in solution and causes detrimental effects on the chaperone binding to the unfolded protein. This is further supported by the observations that a higher amount of DjA1 impaired rather than facilitated the refolding reaction. The DjA1 accelerates the intrinsic ATPase activity of Hsc70 (5), and the simultaneous addition of Hsc70 and DjA1 at folding-compatible concentrations caused detrimental effects on the chaperone binding to the unfolded protein. 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Hsc70 and DjA1 Binding to an Unfolded Protein

The current study revealed three key findings regarding the initial binding process of the Hsc70 components, thus indicating a dynamic mechanism of folding reaction. First, the amount of DjA2 as well as DjA1 could be strikingly decreased to a substoichiometric level with that of the unfolded protein for significant folding (Fig. 1). Intermolecular interactions are disfavored in a diluted condition, and the folding competence of unfolded luciferase was relatively maintained in a second phase (Fig. 1C). This observation prompted an investigation of the chaperone binding to the unfolded protein on a sensor chip by SPR measurement, and the results provided the second key finding. The Hsc70 binding was consistent with a two-state model that allows a step for ATP hydrolysis, whereas the DjA1 binding to a bivalent model indicated a dimer function (Fig. 3). Multiple Hsc70 proteins and a substoichiometric amount of DjA1 dimer independently bound to a substrate protein (Fig. 2). The third key finding was revealed by the screening of the PepSpot peptides (Fig. 5). There were discrete binding sites for the Hsc70 and the DjA1. There was no overlap between the sites for Hsc70 and for DjA1 binding. All of these findings argue against the possible idea that a bound DnaJ protein on a substrate protein recruits and targets the Hsp70 protein. This was further supported by the observation that the initial association rate of Hsc70 was faster than that of DjA1 at folding-compatible concentrations (Fig. 4). Five clusters of consecutive spots as well as some isolated spots gave positive signals (Fig. 5A). The clusters were fewer and shorter than those reported for the bacterial DnaK (12), and most of the clusters overlapped with those for DnaK. The similarity in the binding specificity for DnaK and Hsc70 has also been previously reported (22).

The screening for the DjA1 binding sites was performed according to the published protocol for DnaJ (13). The DjA1 binding gave two clusters of consecutive spots with strong signals (Fig. 5B). These clusters overlapped with those for the bacterial DnaJ (13). The overlap was only four residues (Phe433–Asp436) among the clusters for Hsc70 and DjA1 (Fig. 5, C and D). The identified clusters were scattered along the sequence and denoted in the native structure of luciferase (Fig. 5E).

**TABLE 1**

Kinetic constants for the interaction of chaperones with unfolded luciferase

| Chaperones | $k_{on}$ | $k_{off}$ | $k_{cat}$ |
|------------|----------|-----------|-----------|
| Hsc70$^0$ | 4.9×10$^3$ | 1.0×10$^{-7}$ | 2.1 | 1.0×10$^{-7}$ | 5.8×10$^{-7}$ | 5.6×10$^{-4}$ |
| DjA1$^+$ | 4.0×10$^3$ | 3.7×10$^{-7}$ | 0.91 | 2.5×10$^{-7}$ | 1.9×10$^{-7}$ | 7.7 RU |

$^a$ The $k_{on}$ values were calculated from the $k_{off}$ values.  
$^b$ The responses with 200 μg/ml of Hsc70 were analyzed by the two-state model. The multiplicity of the binding sites was not considered.  
$^c$ The responses with 20 μg/ml of DjA1 were analyzed by the bivalent analyte model.

dependent manner. This simple SPR method yielded new insights into the binding mechanism of chaperone components to a metastable unfolded protein.

**Screening of Binding Peptides for Hsc70 and DjA1**—Cellulose-bound PepSpot peptides were screened to determine the binding site(s) within luciferase sequence recognized by Hsc70 and DjA1. The PepSpot peptides were composed of 13-mers that overlap with adjacent peptides by 10 residues and presented all the potential binding sites to the chaperones. Rüdiger et al. (12, 13) successfully determined binding motifs for the bacterial Hsp70 system with similar peptide arrays. The PepSpot membranes were individually incubated with Hsc70 and DjA1, respectively, to equilibrium. The PepSpot membrane was washed stringently and immunodetected directly because the Hsc70 binding was very tight at equilibrium (Fig. 4). Five clusters of consecutive spots as well as some isolated spots gave positive signals (Fig. 5A). The clusters were fewer and shorter than those reported for the bacterial DnaK (12), and most of the clusters overlapped with those for DnaK. The similarity in the binding specificity for DnaK and Hsc70 has also been previously reported (22).

The screening for the DjA1 binding sites was performed according to the published protocol for DnaJ (13). The DjA1 binding gave two clusters of consecutive spots with strong signals (Fig. 5B). These clusters overlapped with those for the bacterial DnaJ (13). The overlap was only four residues (Phe433–Asp436) among the clusters for Hsc70 and DjA1 (Fig. 5, C and D). The identified clusters were scattered along the sequence and denoted in the native structure of luciferase (Fig. 5E).

**DISCUSSION**

The current study revealed three key findings regarding the initial binding process of the Hsc70 components, thus indicating a dynamic mechanism of folding reaction. First, the amount of DjA2 as well as DjA1 could be strikingly decreased to a substoichiometric level with that of the unfolded protein for significant folding (Fig. 1). Intermolecular interactions are disfavored in a diluted condition, and the folding competence of unfolded luciferase was relatively maintained in a second phase (Fig. 1C). This observation prompted an investigation of the chaperone binding to the unfolded protein on a sensor chip by SPR measurement, and the results provided the second key finding. The Hsc70 binding was consistent with a two-state model that allows a step for ATP hydrolysis, whereas the DjA1 binding to a bivalent model indicated a dimer function (Fig. 3). Multiple Hsc70 proteins and a substoichiometric amount of DjA1 dimer independently bound to a substrate protein (Fig. 2). The third key finding was revealed by the screening of the PepSpot peptides (Fig. 5). There were discrete binding sites for the Hsc70 and the DjA1. There was no overlap between the sites for Hsc70 and for DjA1 binding. All of these findings argue against the possible idea that a bound DnaJ protein on a substrate protein recruits and targets the Hsp70 protein. This was further supported by the observation that the initial association rate of Hsc70 was faster than that of DjA1 at folding-compatible concentrations (Fig. 4).

Hsc70 Binding to the Unfolded Protein—It was surprising to find a large amount of Hsc70 bound to the unfolded protein. The SPR analysis suggested that Hsc70 bound a maximum of
**Hsc70 and DjA1 Binding to an Unfolded Protein**

![Simultaneous addition of chaperone components. A, sensorgrams of the interaction between chaperone combinations and the luciferase (thick curves). ATP, together with the chaperone components, was only added during the association phase. B, differences between the combinational and individual chaperon binding. C, refolding of luciferase with a higher amount of DjA1. The refolding mixtures contained Hsc70 (2.8 μM) and variable amount of DjA1 (0–7.9 μM) with (closed circles) or without (open circles) Bag1 (0.77 μM). The activity was monitored after 60 min. D, refolding of the denatured luciferase on the sensor chip. The coupled luciferase on the sensor chip was manually processed for the unfolding procedure. The chip was immersed in the HKMB-ATP solution with or without the chaperone combination for 30 min and soaked into the luciferase assay buffer to check the activity.

three or four sites (Figs. 2 and 3). This estimation is consistent with the number of clusters that were identified in the peptide screening (Fig. 5). The luciferase polypeptide contains 40 lysine residues, among which nine residues are present in the identified clusters. The amine coupling procedure utilizes a small number of lysine residues within the polypeptide, and this was expected clusters. The amine coupling procedure utilizes a small number of lysine residues within the polypeptide, and this was likely to have detrimental effects on Hsc70 binding and subsequent folding reaction. In any case, several Hsc70 molecules bind to a luciferase molecule in an unfolded state.

The unfolded luciferase gradually lost its binding competence (Fig. 2). The appropriate number of Hsc70 molecules cannot saturate the multiple binding sites at a lower Hsc70 concentration (Fig. 3A), and this results in poor refolding (Fig. 1B). The primary function of Hsc70 appears to be the prevention of inappropriate hydrophobic interactions, and the saturation of the multiple binding sites is necessary for the unfolded protein to reduce the chance of a misfolded conformation (Fig. 6). Therefore, the Hsc70 binding at multiple sites on the unfolded protein is a prerequisite for the refolding reaction. It is generally postulated that multiple Hsp70 molecules bind to a protein and support unidirectional translocation across organelle membranes (23, 24).

The finding that the unfolded protein has multiple sites for Hsc70 binding suggests that a model that takes into consideration the multiple parallel binding reactions with two-state conformational changes on individual binding sites is a more plausible model. However, this model is too complicated to solve based on the current fitting algorithms. Therefore, a practical approach was used to fit the experiment data with a simple “two-state reaction” model. Although this approximation assumes few differences among the affinities of the individual binding sites, the model gave satisfactory results with the statistical values ($x^2 < 2$) and includes the basic features of Hsc70 binding at the initial step. The apparent first dissociation constant of the Hsc70-ATP ($K_{D70}$) was at the micromolar level (Table 1). The $K_{D70}$ value is comparable with the dissociation constants for other Hsp70s and peptide substrates (25, 26) or native $\sigma^{32}$ (27). The association rate constant ($k_{on}$) is a little lower than the $k_{off}$ value reported for DnaK-$\sigma^{32}$ (27).

The association rate of the second step is much slower than that of the first step (Fig. 3 and Table 1). The time required for the half-maximum binding of the second step (Fig. 3C, $t_{1/2} = \sim 17$ min) is very close to the turnover of the ATPase ($t_{1/2} = \sim 20$ min) (5). A slight increase in the binding to the unfolded protein was also observed for the labeled Hsc70 (Fig. 3E, lanes 1 and 2), and it appeared to be more resistant to tryptic digestion (Fig. 3E, lanes 3 and 4). In the absence of the unfolded protein, some tryptic fragments of the Hsc70 in an ADP state were slightly weaker than those in an ATP state (Fig. 3E, lanes 6 and 7). Unfolded proteins accelerate the ATPase activity of Hsp70 in combination with the other components (15). However, the presence of unfolded luciferase had little effect on the ATPase of DnaK without other components (15). The tight binding of Hsc70 in the second step is corroborated by many examples of stable complexes found in Hsc70 and various proteins within the cells (for example, please see Ref. 28).

**Binding of DnaJ Proteins to the Unfolded Protein**—Either of the two type 1 DnaJ proteins, DjA1 and DjA2, was an essential component of the Hsc70 system for the refolding reaction (5). The DjA1 was predominantly dimer and the monomeric species of DjA1 was hardly detected (Fig. 3F). The structural studies revealed that the type 1 DnaJ proteins exist as “U-shaped” dimers (29, 30). The dimeric binding to a substrate protein was suggested for the bacterial DnaJ (31); however, no kinetic evidence of bivalency has been reported for DnaJ protein binding to an unfolded protein. The present study indicated that DjA1 reasonably bound to an unfolded protein bivalently (Fig. 3), and there were two clusters of strong binding sites (Fig. 5). Apart from the issue of the amount of binding, the bivalent model nicely describes the dimeric DjA1 binding. We also found minor species of DjA1 with a high molecular weight (Fig. 3F). The DjA1 protein tends to form aggregates, and it is adsorbed on the tube wall or the column under a low salt buffer. These problems were relieved by the addition of either protein (such as albumin) or a high amount of salt (for instance, 0.5 M NaCl). It is therefore unlikely that these minor species of DjA1 participate in the refolding reaction.

The two binding sites for DjA1 are separated by ~150 residues and were located in two distinct domains (Fig. 5E). Obviously, the bivalent binding considerably restricts the range of conformational transitions for the unfolded protein (Fig. 6), and this would be an important and necessary function for a type 1 DnaJ protein in the refolding reaction.

The DjA1 as well as DjA2 bound to the unfolded luciferase in a substoichiometric manner (Figs. 2 and 3 and supplemental Fig. S4). This contrasts with the finding that the Hsc70 binding showed a higher stoichiometry. This was rather surprising; however, a substoichiometric amount of DjA1 to the unfolded luciferase could permit significant refolding (Fig. 1B).

3 K. Terada, unpublished observation.
was found to be 0.9

It is likely that DjA1 and DjA2 binding to the unfolded protein was comparable with the DjA1 system has little effect on the refolding reaction (4, 5). The DjA2 rate of the folding reaction.

slow saturation of the binding sites for DjA1 determines the process of Hsc70 and DjA1 binding but also suggest that the slow saturation of the binding sites for DjA1 determines the rate of the folding reaction.

The simultaneous addition of DjA1 and DjA2 to the Hsc70 system has little effect on the refolding reaction (4, 5). The DjA2 binding to the unfolded protein was comparable with the DjA1 binding (supplemental Fig. S4). It is likely that DjA1 and DjA2 compete for the same binding sites, and a bound DnaJ protein functions similarly for the refolding reaction. The DjB1, in contrast, was dispensable for the refolding reaction (5), and the SPR measurement showed a small amount of bivalent binding with a high bulk refractive index (supplemental Fig. S4). The high refractive index indicates that this type 2 DnaJ protein associates to the unfolded protein in a rapid and loose manner. This feature may promote recruitment of Hsc70 to newly synthesized proteins for the de novo folding (2) and accelerate the conversion of the bound Hsc70-ATP into the Hsc70-ADP. Accordingly, the direct binding of the DnaJ proteins was not observed for the nascent polypeptides (32). The physiological amount of DjB1 showed little bivalent binding (supplemental Fig. S4). This may be why a larger amount of DjB1 and an extra addition of the reticulocyte lysate was necessary for efficient refolding (33). The DjB1 is not the prime DnaJ protein for the refolding of the unfolded protein (4, 5), and the DjB1 knock-out animal showed no obvious abnormal phenotype under normal conditions (34).

**Initial Recognition of Substrate Proteins by Hsc70 Components**—The SPR measurements of individual chaperone binding in folding-compatible concentrations demonstrated that the initial association rate of DjA1 was slower than that of the Hsc70. Attenuated binding was observed with the simultaneous addition of Hsc70 and DjA1, but it was apparently improved by further addition of Bag1 (Fig. 4). A DnaJ protein promotes hydrolysis of the ATP bound to the Hsc70 and generates ADP-bound Hsc70, whereas Bag1 exchanges bound ADP to ATP and restored the population of ATP-bound Hsc70 (35). Furthermore, a DnaJ protein complexes with Hsp70-ATP in solution with dissociation constants at the submicromolar level (21, 36), whereas the Bag1 complexes with Hsc70 at a dissociation constant of 1–3 μM (37). Therefore, the effective concentrations of the Hsc70-ATP and DnaJ protein that can bind to the unfolded protein differ significantly from the original concentrations of the components. They are dependent on the kinds, ratio, and concentrations of the constituted components.

The postulated roles for DnaJ protein in targeting of Hsp70 and transferring of substrate to the Hsp70 are not always operable in the refolding of luciferase. The conformational changes in the inactivation process of α22 led to a similar conclusion by the bacterial DnaK-DnaJ system (38). Native α22 appears to have two distinct binding sites for DnaK and DnaJ. Therefore, the canonical type 1 DnaJ proteins bind to distinct sites on the substrates without subsequent transferring of the bound substrate to Hsp70 proteins (38). Some noncanonical DnaJ pro-
teins (types 2 and 3) are membrane-anchored and engage in protein translocation across organelle membranes (16). Although the proximal effect of a DnaJ protein for recruiting an Hsp70 was reported previously (39), the transferring of the DnaJ-bound site to Hsp70 is remained to be determined. In any case, distinct binding mechanisms may exist among the Hsp70 systems dependent upon the type of accompanying DnaJ protein.

The current findings emphasize the importance of interactions between an unfolded protein and chaperones. Although the refolding on a sensor chip seems not to be perfect, the SPR analysis demonstrated important features of the unfolded protein recognition by the Hsc70 system at the initial step. Furthermore, they provide insights into the mechanistic action of the DnaJ protein among various cochaperone-mediated biological processes of the other Hsp70 systems. The substrate polypeptides have diverse fates on the biological processes. Some have an appropriate binding site(s) to be mediated by the canonical DnaJ protein as an Hsc70 system. However, slow and substoichiometric binding of DjA1 to the unfolded protein suggests that the remaining Hsc70-bound but DjA1-unbound substrates have a good chance to be recognized by either other DnaJ proteins or other cochaperones that have tetratricopeptide repeat domains. The tetratricopeptide repeat-containing cochaperones bind to the EEVD-terminal sequence of Hsc70 and modulate the Hsc70 system to engage in other biological processes besides folding (40–42).

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