

Distinct Roles of the N-terminal-binding Domain and the C-terminal-solubilizing Domain of α -Synuclein, a Molecular Chaperone*

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α -Synuclein, an acidic neuronal protein of 140 amino acids, is extremely heat-resistant and is natively unfolded. Recent studies have demonstrated that α -synuclein has chaperone activity both *in vitro* and *in vivo*, and that this activity is lost upon removing its C-terminal acidic tail. However, the detailed mechanism of the chaperone action of α -synuclein remains unknown. In this study, we investigated the molecular mechanism of the chaperone action of α -synuclein by analyzing the roles of its N-terminal and C-terminal domains. The N-terminal domain (residues 1–95) was found to bind to substrate proteins to form high molecular weight complexes, whereas the C-terminal acidic tail (residues 96–140) appears to be primarily involved in solubilizing the high molecular weight complexes. Because the substrate-binding domain and the solubilizing domain for chaperone function are well separated in α -synuclein, the N-terminal-binding domain can be substituted by other proteins or peptides. Interestingly, the resultant engineered chaperone proteins appeared to display differential efficiency and specificity in terms of the chaperone function, which depended upon the nature of the binding domain. This finding implies that the C-terminal acidic tail of α -synuclein can be fused with other proteins or peptides to engineer synthetic chaperones for specific purposes.

α -Synuclein, a major constituent of Lewy bodies in Parkinson's disease, is an acidic neuronal protein that is composed of 140 amino acids (1–4). α -Synuclein is extremely heat-resistant and natively unfolded with an extended structure primarily composed of random coils (5–7). α -Synuclein consists of three distinct regions (reviewed in Refs. 8–11). The N-terminal region of α -synuclein contains KTKEGV repeats, which form amphipathic α -helices that are reminiscent of the lipid-binding domain of apolipoproteins (12). The central region of α -synuclein is composed of a very hydrophobic non-A β component of Alzheimer's disease amyloid (NAC)¹ peptide, and the

acidic C-terminal region of α -synuclein is composed primarily of acidic amino acids. Moreover, the amphipathic N-terminal and the hydrophobic NAC regions are highly conserved between species, whereas the C-terminal region is highly variable in size and in sequence (reviewed in Refs. 8–11). In addition to α -synuclein, the β - and γ -synucleins and synoretin, members of the synuclein family, have been identified in humans (1, 2, 13, 14).

α -Synuclein has been suggested to be implicated in the pathogenesis of Parkinson's disease and related neurodegenerative disorders (reviewed in Refs. 8–11), and more recently, to be an important regulatory component of vesicular transport in neuronal cells (15). Moreover, α -synuclein has been suggested to function as a chaperone protein *in vivo* because it appears to bind many cellular proteins (16–22). In particular, α -synuclein shares regions of homology with 14-3-3 proteins (18), which are a family of ubiquitous cytoplasmic chaperones (23), and binds to 14-3-3 proteins as well as to the ligands of 14-3-3 including PKC, BAD, and ERK (18). More importantly, α -synuclein is overexpressed under stress conditions (18). Recently, the chaperone activity of α -synuclein *in vitro* has been demonstrated by two research groups (24, 25). Like other small heat shock proteins (sHSPs), such as HSP25, HSP16, and α -crystallin (26–31), α -synuclein is able to prevent the thermally and chemically induced aggregation of substrate proteins. The other synuclein family members, the β - and γ -synucleins, also appear to have this chaperone activity (24). However, the detailed mechanism of the chaperone action of α -synuclein remains unknown. Interestingly, the chaperone activity of α -synuclein is lost upon removing its C-terminal acidic tail (24), suggesting that the acidic tail plays an important role in the molecular chaperone function. Furthermore, conformational changes induced in α -synuclein by environmental factors and its consequent aggregation abolish the chaperone activity of α -synuclein (25), which suggests that the natively unfolded conformation might be essential for the substrate binding and subsequent stabilization by α -synuclein.

The mechanism of the chaperone action of sHSPs, on the other hand, is comparatively well understood. sHSPs protect substrate proteins from stress (*e.g.* heat, chemicals, etc.) by forming high molecular weight (HMW) complexes with partially unfolded substrate proteins (27, 32–40). However, alone sHSPs do not have the ability to protect enzymes from thermal inactivation or to promote their functional refolding after de-

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¹ The abbreviations used are: NAC, non-A β component of Alzheimer's disease amyloid; GST, glutathione S-transferase; HMW complex, high

molecular weight complex; sHSP, small heat shock protein; DHFR, dihydrofolate reductase; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; PBS, phosphate-buffered saline.

naturation (31, 39, 41, 42), although a few exceptional cases with marginal effects have been reported (30, 33, 43–46). sHSPs have, therefore, been classified as “junior chaperones” (47). sHSPs share many properties, for example, they have extensive amino acid sequence similarity, and are found as large, aggregated complexes of average mass 200–800 kDa (reviewed in Refs. 48 and 49). The charged C-terminal domain (also called the α -crystallin domain) is well conserved in all members of the sHSP family, whereas the hydrophobic N-terminal domain is variable in length and sequence (49). The N-terminal domain is known to play a crucial role in self-assembly and thus contributes to chaperone activity (38, 50), whereas the C-terminal domain is known to be crucial for substrate protein binding and stabilization (51). In particular, a 19-amino acid peptide derived from the C-terminal domain has been shown to possess substantial chaperone activity (52). The extended polar C-terminal tail (10–18 amino acid residues) is also important in the chaperone action of sHSPs, and appears to fulfill many roles that are not completely understood yet. First, the extended C-terminal tail of sHSPs is believed to function as a solubilizer (26, 53–56). Moreover, truncation of the C-terminal tail results in a significant decrease in the chaperone function and stability of sHSPs (55, 57). In some sHSPs, the flexible C-terminal tail also appears to interact directly with substrate proteins (29, 54). Furthermore, the crystal structure of HSP16 reveals that the C-terminal tail is also involved in the organization of the HSP oligomer (27, 28).

In this study, we investigated the molecular mechanism of the chaperone action of α -synuclein by analyzing the roles of the N-terminal and C-terminal domains of α -synuclein in the molecular chaperone function. Unlike the sHSPs, the substrate-binding domain and the solubilizing domain for chaperone function appeared to be well separated in α -synuclein.

EXPERIMENTAL PROCEDURES

Materials—GSH, dithiothreitol (DTT), 1-chloro-2,4-dinitrobenzene, and isopropyl- β -D-thiogalactopyranoside were purchased from Sigma. Glutathione-Sepharose 4B was obtained from Pepton (Taejon, Korea). Bovine plasma thrombin was supplied by Sigma. Leupeptin, pepstatin, and phenylmethylsulfonyl fluoride were purchased from Roche Molecular Biochemicals. Aldolase from rabbit muscle, insulin from the bovine pancreas, lysozyme from chicken eggs, and luciferase from the firefly (*Photinus pyralis*) were purchased from Sigma.

Purification of α -Synuclein Deletion Mutants and GST-Synuclein Fusion Proteins— α -Synuclein was overexpressed in *Escherichia coli* and the recombinant protein was purified to apparent homogeneity by taking advantage of the thermosolubility of the protein and by using conventional column chromatography, as described previously (6, 58). The GST protein encoded by the pGEX expression vector was purified by affinity chromatography using glutathione-Sepharose 4B beads, and further purified on an FPLC gel-filtration column. The GST- α -synuclein fusion constructs, pGST-Syn-(61–140) and pGST-Syn-(96–140), were generated by the PCR amplification of the α -synuclein gene with specific primer sets containing the *Bgl*II and *Sal*I restriction sites (59). The GST-synuclein fusion constructs were transformed into the *E. coli* strain, BL21(DE3), and the recombinant GST-synuclein fusion proteins, GST-Syn-(61–140) and GST-Syn-(96–140), were purified by affinity chromatography using glutathione-Sepharose 4B beads. The GST-synuclein fusion proteins were further purified on an FPLC gel-filtration column. The α -synuclein deletion mutants, Syn-(61–140) and Syn-(96–140), were prepared from GST-Syn-(61–140) and GST-Syn-(96–140), respectively, by thrombin digestion of the fusion proteins.

Construction of DHFR-Syn-(96–140) Expression Vector—The protein coding region of dihydrofolate reductase (DHFR) was subcloned into an *E. coli* expression vector, pRSETA, using *Bam*HI and *Hind*III restriction sites (pDHFR). The protein coding region of the C-terminal acidic tail of α -synuclein (residues 96–140) was amplified by PCR with the 5'-oligonucleotide primer GCGCGGTACCAAGGACCAAGTTGGCCAA-GAATG containing the underlined *Kpn*I restriction site and 3'-oligonucleotide primer GCGCGTCACTTAGGCTTCAGGTTCGTAGT containing the underlined *Sal*I restriction site. The amplified DNAs were gel purified, digested with appropriate enzymes, then ligated into the

pDHFR vector that had been digested with the appropriate restriction enzymes and gel purified. All constructs were verified by DNA sequencing.

Bacterial Expression and Purification of DHFR-Syn-(96–140)—The DHFR-Syn-(96–140) fusion construct was transformed into the *E. coli* strain BL21(DE3) for protein expression. The transformed bacteria were grown in a LB medium with 0.1 mg/ml ampicillin at 37 °C to an A_{600} of 0.8, then cultured for a further 4 h after being induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside. The cells were harvested by centrifugation at 10,000 rpm for 10 min, resuspended in phosphate-buffered saline (PBS, pH 7.4), then disrupted by ultrasonication. After removing the cell debris, the supernatants were loaded onto a nickel-nitrilotriacetic acid column equilibrated with a loading buffer (50 mM phosphate buffer (pH 8.0) containing 0.3 M NaCl and 10 mM imidazole). After washing with the loading buffer, the protein was eluted with 250 mM imidazole in the same buffer. The DHFR-Syn-(96–140) fusion protein was further purified on an FPLC gel-filtration column. The protein was concentrated and the buffer changed by Centricon (Amicon, Beverly, MA).

Chaperone-like Activity Assay—The ability of chaperone proteins to prevent heat-induced aggregation of substrate proteins (GST and aldolase) was monitored as described previously (25). Briefly, substrate proteins (0.2 mg/ml as a final concentration) in PBS (pH 7.4) were incubated with each chaperone protein at 65 °C for specified times (see figure legends) in a cuvette. Light scattering was then monitored at 360 nm as a function of time, using a spectrophotometer (Beckman DU-650). The ability of the chaperone proteins to prevent chemically induced substrate protein (insulin and lysozyme) aggregation was monitored as described previously (24, 25). Substrate proteins (0.5 mg/ml as a final concentration) in 10 mM phosphate buffer (pH 7.4) were incubated with the indicated amounts of each chaperone protein at room temperature (see figure legends). DTT was added, to a final concentration of 20 mM, to commence the denaturation and precipitation of substrate proteins. Light scattering was then monitored at 360 nm using a spectrophotometer (Beckman DU-650). In addition, luciferase (0.1 mg/ml in PBS as a final concentration) was incubated with each chaperone protein for 10 min at 65 °C in a cuvette, and light scattering was monitored at 360 nm (Fig. 6B). For the insulin aggregation assay in Fig. 6A, insulin alone (0.5 mg/ml as a final concentration in 10 mM phosphate buffer, pH 7.4) or a mixture of insulin and GST-Syn-(96–140) (0.5 mg/ml each as a final concentration) was preincubated for 5 min at 59 °C in a thermostatic cell holder, and this temperature was maintained during the chaperone assay. After adding 2 mM DTT as a final concentration, the absorbance was measured at 360 nm as a function of time.

Gel-filtration Chromatographic Analysis of the High Molecular Weight Complexes—Individual solutions of each chaperone protein and substrate protein (GST or aldolase, final concentration of 0.1–0.2 mg/ml in PBS), or mixtures of chaperone and substrate proteins (final concentration of 0.2–0.5 mg/ml chaperone protein with 0.1–0.2 mg/ml substrate protein in PBS) were either heat treated (65 °C for 10 min) or not heat-treated, and then centrifuged for 10 min at 13,000 rpm to remove precipitated proteins. 500 μ l of each supernatant was loaded onto the Superdex 75 HR 10/30 column (Amersham Biosciences) equilibrated in PBS (pH 7.4), and the proteins were eluted at a flow rate of 1 ml/min at room temperature. Fractions corresponding to each protein peak were collected and analyzed in 12% SDS-polyacrylamide gels. To detect α -synuclein in the HMW complex, 25 μ l of each fraction was loaded into a 12% SDS-polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane, and Western blotted with rabbit polyclonal anti- α -synuclein antibodies.

GST Activity Assay—The enzymatic activity of GST was assayed using a chromogenic substrate, 1-chloro-2,4-dinitrobenzene, as described previously (60). The GST enzyme was added to the substrate solution (1 mM GSH and 2 mM 1-chloro-2,4-dinitrobenzene in 0.1 M phosphate buffer, pH 7.4) to a final concentration of 20 μ g/ml and incubated at 37 °C for 10 min. Enzyme activity was measured as an increased absorbance at 350 nm, corresponding to the maximum absorbance of 1-S-glutathionyl-2,4-dinitrobenzene, using the Spectramax 250 microplate reader (Molecular Devices, Menlo Park, CA).

Phosphatase Activity Assay—The catalytic activity of protein-tyrosine phosphatase-1B was assayed at 37 °C for 60 min in a reaction mixture (0.2 ml) containing 10 mM *p*-nitrophenyl phosphate as substrate. The buffer used was 20 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA, and 2 mM DTT. The reaction was initiated by adding enzyme and quenched after 60 min by the addition of 1 ml of 1 N NaOH. The amount of *p*-nitrophenol released was determined by measuring the absorbance at 405 nm.

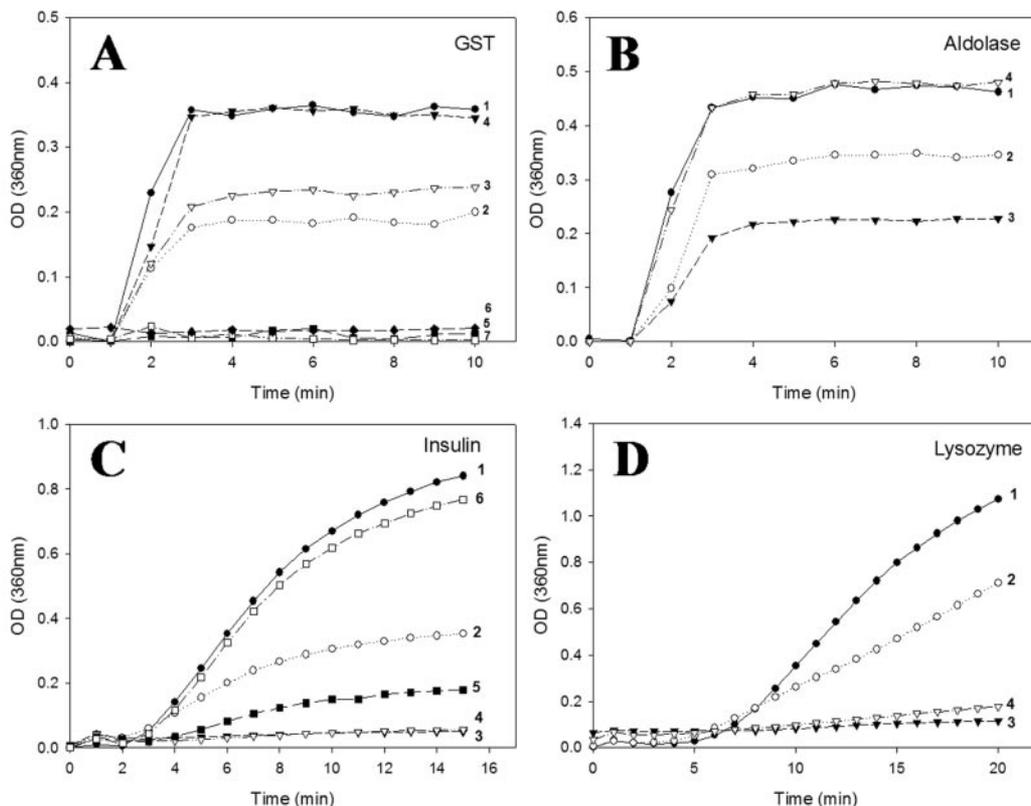


FIG. 1. Chaperone-like activity of α -synuclein and its deletion mutants. A and B, thermally induced aggregation assay. A, aggregation curves of GST (0.2 mg/ml in PBS, pH 7.4) at 65 °C in the absence and presence of α -synuclein deletion mutants. Lines: 1, GST alone; 2, GST + α -synuclein (0.2 mg/ml); 3, GST + Syn-(61–140) (0.2 mg/ml); 4, GST + Syn-(96–140) (0.2 mg/ml); 5, α -synuclein (0.2 mg/ml) alone; 6, Syn-(61–140) (0.2 mg/ml) alone; 7, Syn-(96–140) (0.2 mg/ml) alone. B, aggregation curves of aldolase (0.2 mg/ml in PBS, pH 7.4) at 65 °C in the absence and presence of α -synuclein deletion mutants. Lines: 1, aldolase alone; 2, aldolase + α -synuclein (0.2 mg/ml); 3, aldolase + Syn-(61–140) (0.2 mg/ml); 4, aldolase + Syn-(96–140) (0.2 mg/ml). C and D, DTT-induced aggregation assay. C, aggregation curves of insulin (0.5 mg/ml in 10 mM phosphate buffer, pH 7.4) induced with 20 mM DTT in the absence and presence of α -synuclein deletion mutants. Lines: 1, insulin alone; 2, insulin + α -synuclein (0.5 mg/ml); 3–5, insulin + Syn-(61–140) (1:1, 1:0.5, and 1:0.1, w/w, respectively); 6, insulin + Syn-(96–140) (0.5 mg/ml). D, aggregation curves of lysozyme (0.5 mg/ml in 10 mM phosphate buffer, pH 7.4) induced with 20 mM DTT in the absence and presence of α -synuclein deletion mutants. Lines: 1, lysozyme alone; 2, lysozyme + α -synuclein (1:1, w/w); 3 and 4, lysozyme + Syn-(61–140) (1:1 and 1:0.2, w/w, respectively).

RESULTS

The C-terminal Acidic Tail Is Necessary, but Insufficient for the Chaperone Activity of α -Synuclein—Previous studies have shown that α -synuclein has chaperone activity *in vitro* (24, 25). Like other small molecular chaperone proteins, α -synuclein was able to protect a variety of proteins from stress-induced aggregation. Interestingly, the chaperone activity of α -synuclein was completely lost upon removing its C-terminal acidic tail (24), which suggests that the acidic tail was critical for its chaperone activity. We first asked whether the acidic tail was sufficient to have the chaperone activity. To address this question, we constructed two deletion mutants encoding either the acidic tail only (residues 96–140, Syn-(96–140)) or the NAC region plus the acidic tail (residues 61–140, Syn-(61–140)), and compared their chaperone activities with that of wild type α -synuclein. The effect of the deletion mutants on the heat-induced precipitation of substrate proteins was first investigated by using the conventional chaperone activity assay. As shown in Fig. 1A, the C-terminal acidic tail alone (Syn-(96–140)) did not protect GST from heat-induced precipitation (Fig. 1A, line 4), whereas Syn-(61–140) containing the NAC region and the acidic tail appeared to protect GST from heat-induced precipitation almost as effectively as the wild type α -synuclein (Fig. 1A, lines 3 and 2, respectively). This result suggests that Syn-(61–140) has all the units necessary for chaperone activity. Similar results were obtained when aldolase was used as the substrate for the chaperone activity assay (Fig. 1B).

We next compared the chaperone activity of the α -synuclein

deletion mutants by measuring the chemically induced aggregation of insulin and lysozyme (Fig. 1, C and D). Consistent with the results obtained from the heat-induced precipitation assay, Syn-(96–140) did not protect the substrate proteins from DTT-induced precipitation (Fig. 1C, line 6). On the other hand, Syn-(61–140) effectively protected the substrate proteins from DTT-induced precipitation (Fig. 1, C, lines 3–5, and D, lines 3 and 4). Interestingly, Syn-(61–140) appeared to be much more efficient than wild type α -synuclein (Figs. 1, C and D, line 2) in terms of protecting the substrate proteins from DTT-induced precipitation. This aggregation was almost completely suppressed at an insulin to chaperone weight ratio of 1:0.5, corresponding to a stoichiometric ratio of 1:0.3 (Fig. 1C, line 4). Syn-(61–140) also appeared to protect lysozyme from DTT-induced aggregation at a substoichiometric ratio (Fig. 1D, line 4). Syn-(61–140), Syn-(96–140), and wild type α -synuclein alone did not precipitate under these conditions (data not shown). These results indicate that the C-terminal acidic tail of α -synuclein was necessary, but not sufficient for the chaperone activity of α -synuclein, and also suggest that the N-terminal region (residues 1–95) of α -synuclein may determine the efficiency of the chaperone function.

The N-terminal Region Mediates Substrate Protein Binding—Small molecular chaperones, such as small heat shock proteins, α -crystallin, tubulin, and clusterin, prevent protein precipitation by forming soluble HMW complexes (27, 32–40). We first confirmed that α -synuclein also acts in this way to prevent protein precipitation, and then investigated which re-

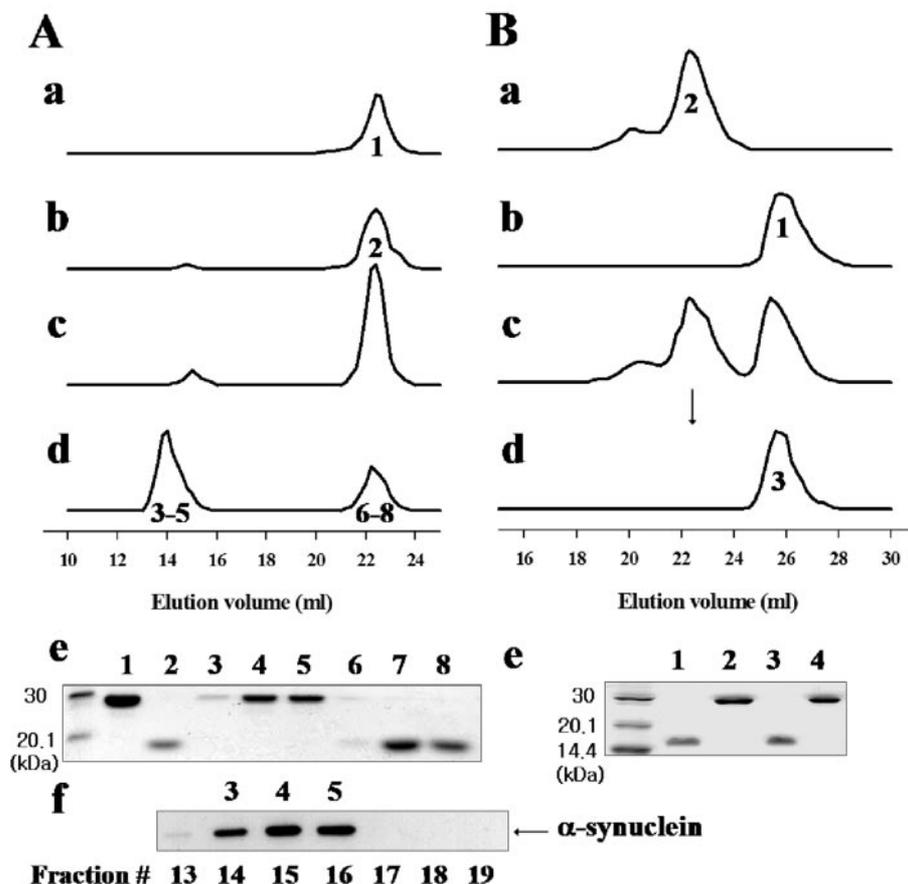


FIG. 2. The N-terminal region of α -synuclein mediates substrate protein binding. A, FPLC gel-filtration chromatography (a–d) and SDS-PAGE (e and f) analysis of the HMW complex of wild type α -synuclein and GST. Protein samples (500 μ l) were loaded onto the Superdex 75 HR column (Amersham Biosciences) equilibrated in PBS, and eluted at a flow rate of 1 ml/min at room temperature. a, GST (0.1 mg/ml). b, α -synuclein (0.5 mg/ml). α -Synuclein incubated at 65 °C for 10 min was eluted at the same position (data not shown). c, GST (0.1 mg/ml) + α -synuclein (0.5 mg/ml) were mixed and loaded onto the column. d, GST (0.1 mg/ml) + α -synuclein (0.5 mg/ml) were mixed and incubated for 10 min at 65 °C and then loaded onto the column. e, SDS-PAGE analysis of the α -synuclein-GST complex. The peak fractions numbered in a–d were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. f, Western blot was performed to detect α -synuclein in the HMW complex (containing peak fractions 3–5 from d). B, FPLC gel-filtration chromatography (a–d) and SDS-PAGE (e) analysis of the Syn-(96–140)-GST complex. a, GST (0.2 mg/ml). b, Syn-(96–140) (0.2 mg/ml). c, GST (0.2 mg/ml) + Syn-(96–140) (0.2 mg/ml) were mixed and loaded onto the column. d, GST (0.2 mg/ml) + Syn-(96–140) (0.2 mg/ml) were mixed and incubated for 10 min at 65 °C and then loaded onto the column. The arrow indicates the expected position for GST. e, SDS-PAGE analysis of the Syn-(96–140)-GST complex. The peak fractions numbered in a–d were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lanes: 1, Syn-(96–140); 2, GST; 3, peak 3 from d; 4, pellet fraction obtained after incubating the Syn-(96–140)/GST mixture for 10 min at 65 °C.

gion of α -synuclein was critical for substrate protein binding (Fig. 2). The substrate protein (GST) was incubated with wild type α -synuclein or its C-terminal fragment (Syn-(96–140)) for 10 min at 65 °C and the protein mixtures were purified on a FPLC gel-filtration column. Each peak fraction was then analyzed on SDS-polyacrylamide gels. As expected, α -synuclein formed a soluble HMW complex with the substrate protein (Fig. 2A). Syn-(61–140) also appeared to form a HMW complex with GST (data not shown). In contrast, the C-terminal acidic tail of α -synuclein (Syn-(96–140)) did not form a HMW complex (Fig. 2B), which suggested that substrate protein binding may be mediated by the N-terminal region of α -synuclein. Unlike other small molecular chaperone proteins, however, the HMW complex formed between α -synuclein and GST contained only a trace amount of α -synuclein, which was detected by immunoblotting (Fig. 2A, f). The immunoreactivity of α -synuclein was only detected in fractions corresponding to the HMW complex, indicating that these HMW forms of α -synuclein were not contaminated by the aggregates. A similar phenomenon was observed when other substrate proteins were used (data not shown). Previous studies have shown that Syn-(1–97) precipitates upon heat treatment and does not have the chaperone activity (24). In addition, NAC and the C-terminal truncated

α -synuclein have been shown to aggregate faster than the full-length α -synuclein (61–64). Taken together, it is highly likely that the N-terminal region of α -synuclein functions as a binding domain for substrate proteins and that the C-terminal acidic tail functions as a solubilizing domain for the HMW complexes and for α -synuclein itself.

The Binding Domain Can Be Substituted by Other Proteins—This study shows that the binding domain and solubilizing domain were structurally distinct in α -synuclein. Based on this finding, we hypothesized that the binding domain could be substituted by other proteins, because the binding domain of molecular chaperone proteins was not likely to be specific for individual substrate proteins. The chaperone activity of Syn-(61–140) (Fig. 1) supports this idea. To prove this hypothesis further, we constructed a GST-synuclein fusion protein, GST-Syn-(96–140), containing the acidic tail of α -synuclein at the C terminus of GST. Surprisingly, GST-Syn-(96–140) prevented GST and aldolase from heat-induced precipitation (Fig. 3, A and B). Furthermore, GST-Syn-(96–140) appeared to be a more efficient chaperone protein than wild type α -synuclein and Syn-(61–140) (Figs. 1, A and B, 3, A and B), and almost completely prevented GST and aldolase from heat-induced precipitation when they were incubated at a ratio of 1:1 (w/w) (Fig. 3,

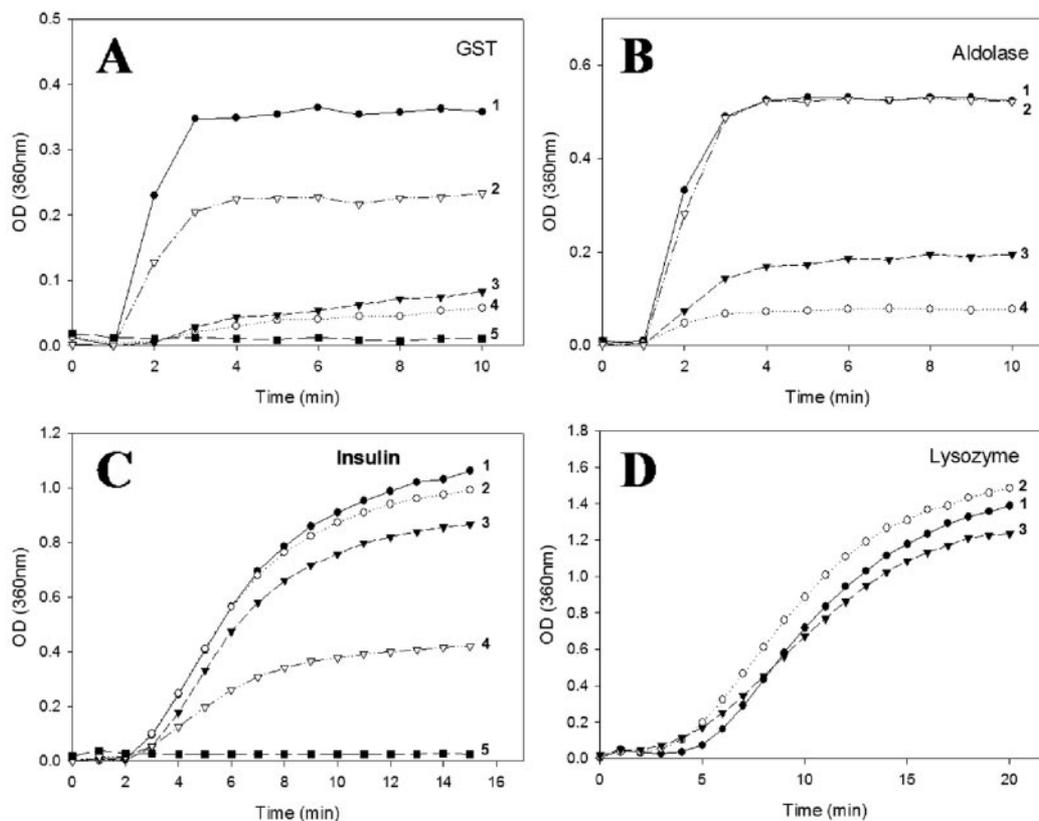


FIG. 3. Chaperone-like activity of GST-Syn-(96-140). A and B, thermally induced aggregation assay. A, aggregation curves of GST (0.2 mg/ml in PBS, pH 7.4) at 65 °C in the absence and presence of GST-Syn-(96-140). Lines: 1, GST alone; 2-4, GST + GST-Syn-(96-140) (1:0.1, 1:0.5, and 1:1, w/w, respectively); 5, GST-Syn-(96-140) (0.2 mg/ml) alone. B, aggregation curves of aldolase (0.2 mg/ml in PBS, pH 7.4) at 65 °C in the absence and presence of GST-Syn-(96-140). Lines: 1, aldolase alone; 2-4, aldolase + GST-Syn-(96-140) (1:0.2, 1:0.5, and 1:1, w/w, respectively). C and D, DTT-induced aggregation assay. C, aggregation curves of insulin (0.5 mg/ml in 10 mM phosphate buffer, pH 7.4) induced with 20 mM DTT in the absence and presence of GST-Syn-(96-140). Lines: 1, insulin alone; 2-4, insulin + GST-Syn-(96-140) (1:1, 1:3, and 1:5, w/w, respectively); 5, GST-Syn-(96-140) (0.5 mg/ml) alone. D, aggregation curves of lysozyme (0.5 mg/ml in 10 mM phosphate buffer, pH 7.4) induced with 20 mM DTT in the absence and presence of GST-Syn-(96-140). Lines: 1, lysozyme alone; 2 and 3, insulin + GST-Syn-(96-140) (1:1 and 1:3, w/w, respectively).

A, line 4, and B, line 4). However, GST-Syn-(96-140) was not as effective as α -synuclein at protecting proteins from chemically induced precipitation (Fig. 3, C and D). In particular, GST-Syn-(96-140) did not prevent lysozyme from DTT-induced precipitation, although it slightly alleviated the DTT-induced precipitation of insulin.

Like other small molecular chaperone proteins, GST-Syn-(96-140) formed a HMW complex when incubated with aldolase, a substrate protein, for 10 min at 65 °C (Fig. 4). GST-Syn-(96-140) also formed a HMW complex with GST when the proteins were co-incubated at 65 °C (data not shown). Unlike α -synuclein, GST-Syn-(96-140) appeared to form HMW complexes with a stoichiometric ratio (Fig. 4, d and f, lanes 3-6), which suggests that it interacts with substrate proteins in a similar way to the sHSPs. These results indicate that the binding domain of α -synuclein can be substituted with another protein or peptide and the resultant engineered protein functions as a molecular chaperone with a different efficiency and specificity.

DHFR-Syn-(96-140) Also Has Chaperone Activity—To address whether any proteins containing the acidic tail of α -synuclein have chaperone activity, we constructed a DHFR-synuclein fusion protein, DHFR-Syn-(96-140), which contains the acidic tail of α -synuclein at the C terminus. Interestingly, DHFR-Syn-(96-140) was extremely heat resistant, whereas DHFR was so heat-labile that it easily precipitated by thermal stress (data not shown). Similar phenomenon has been observed when GST was fused with the acidic tail of α -synuclein (59). We next examined the chaperone activity of DHFR-Syn-

(96-140). As shown in Fig. 5, DHFR-Syn-(96-140) effectively protects aldolase from heat-induced aggregation, indicating that the fusion protein functions as a molecular chaperone. DHFR-Syn-(96-140) also appeared to prevent GST from heat-induced precipitation (data not shown). Therefore, it is highly likely that the C-terminal acidic tail of α -synuclein can be used to engineer synthetic chaperones.

The Binding Domain Determines the Efficiency and Specificity of Chaperone Function—Our data show that engineered chaperone proteins (Syn-(61-140), GST-Syn-(96-140), and DHFR-Syn-(96-140)) containing the acidic tail of α -synuclein as a solubilizing domain display different chaperone activities and substrate specificities, and that these differences might originate from the intrinsic properties of the binding domain. For example, Syn-(61-140) appeared to be a better chaperone than wild type α -synuclein at preventing the DTT-induced precipitation of substrate proteins (Fig. 1, C and D). Furthermore, GST-Syn-(96-140) appeared to inhibit the heat-induced precipitation of substrate proteins far more so than wild type α -synuclein or Syn-(61-140) (Fig. 3, A and B), but to only weakly suppress the DTT-induced precipitation of substrate proteins (Fig. 3, C and D). To confirm the notion that the binding domain might determine the efficiency and specificity of the chaperone function, we induced conformational changes in GST-Syn-(96-140) by heating, and examined changes in its chaperone-like activity during the DTT-induced aggregation of insulin. The conformation of GST-Syn-(96-140) was irreversibly changed at high temperatures with a melting temperature (T_m) of 62 °C (59), but insulin alone did not precipitate at this

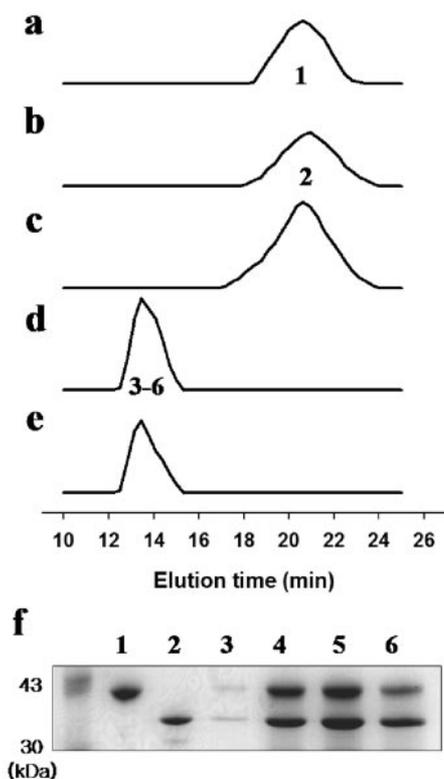


FIG. 4. FPLC gel-filtration chromatography (a-e) and SDS-PAGE (f) analysis of the HMW complex between GST-Syn(96-140) and aldolase. Protein samples (500 μ l) were loaded onto the Superdex 75 HR column (Amersham Biosciences) equilibrated in PBS, and eluted at a flow rate of 1 ml/min at room temperature. *a*, aldolase (0.2 mg/ml). *b*, GST-Syn(96-140) (0.2 mg/ml). *c*, aldolase (0.2 mg/ml) and GST-Syn(96-140) (0.2 mg/ml) were mixed and loaded onto the column. *d*, aldolase (0.2 mg/ml) and GST-Syn(96-140) (0.2 mg/ml) were mixed and incubated for 10 min at 65 $^{\circ}$ C and then loaded onto the column. *e*, GST-Syn(96-140) (0.2 mg/ml) were incubated for 10 min at 65 $^{\circ}$ C and then loaded onto the column. *f*, SDS-PAGE analysis of the HMW complex. The peak fractions numbered in *a-d* were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250.

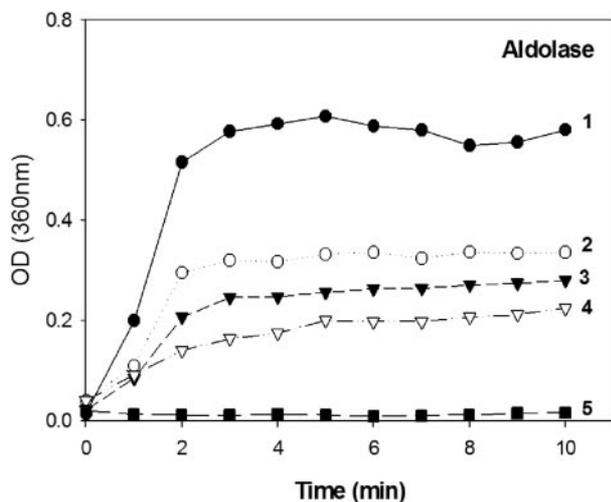


FIG. 5. Chaperone-like activity of DHFR-Syn(96-140). Aggregation curves of aldolase (0.2 mg/ml in PBS, pH 7.4) at 65 $^{\circ}$ C in the absence and presence of DHFR-Syn(96-140). *Lines*: 1, aldolase alone; 2-4, aldolase + DHFR-Syn(96-140) (1:0.5, 1:1, and 1:2, w/w, respectively); 5, DHFR-Syn(96-140) (0.2 mg/ml) alone.

temperature (data not shown). Interestingly, the chaperone activity of GST-Syn(96-140) in the DTT-induced aggregation of insulin was significantly improved at 59 $^{\circ}$ C (Fig. 6A) com-

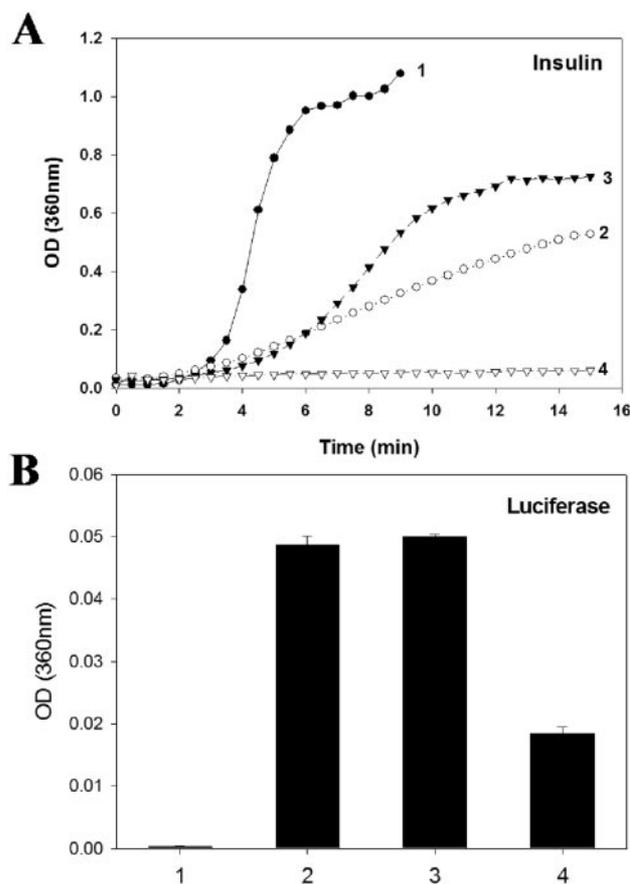


FIG. 6. Substrate specificity of GST-Syn(96-140) as a molecular chaperone. *A*, temperature effect on the chaperone-like activity of GST-Syn(96-140). Protein mixtures were preincubated for 5 min at 59 $^{\circ}$ C, then 2 mM DTT was added to induce the aggregation of insulin, and mixtures were then placed on a thermostatic cell holder. *Lines*: 1, insulin (0.5 mg/ml) alone; 2, insulin + GST-Syn(96-140) (1:1, w/w); 3, insulin + GST-Syn(96-140) (1:0.5, w/w); and 4, GST-Syn(96-140) (0.5 mg/ml) alone. *B*, GST-Syn(96-140) did not protect luciferase from heat-induced aggregation. Protein mixtures were incubated for 10 min at 65 $^{\circ}$ C, and light scattering was measured at 360 nm. *Graphs*: 1, luciferase (0.1 mg/ml) alone at room temperature; 2, luciferase (0.1 mg/ml) alone at 65 $^{\circ}$ C; 3, luciferase + GST-Syn(96-140) (1:1, w/w) at 65 $^{\circ}$ C; 4, luciferase + Syn(61-140) (1:1, w/w) at 65 $^{\circ}$ C.

pared with the case at room temperature (Fig. 3C), suggesting that the efficiency of its chaperone action was affected by conformational changes in its N-terminal substrate-binding domain (GST domain in this case). In addition, we found that GST-Syn(96-140) did not protect the heat-induced aggregation of luciferase, whereas α -synuclein and Syn(61-140) effectively prevented this aggregation (Fig. 6B). Therefore, it seems highly likely that the binding domain determines the efficiency and specificity of the chaperone function.

GST-Syn(96-140) Does Not Protect Enzymes from Heat-induced Inactivation—Small molecular chaperone proteins are generally known to be inefficient at preventing the thermal inactivation of enzymes (31, 39-42, 65), although some small molecular chaperone proteins are reported to have a marginal potential to protect certain enzymes from thermal inactivation (30, 33, 43-47). Previously, we showed that α -synuclein did not protect GST enzyme from heat-induced inactivation (25). To investigate whether GST-Syn(96-140) was able to protect enzymes from thermal inactivation, the thermostabilities of GST and protein-tyrosine phosphatase-1B were measured using thermal inactivation curves in the presence and in the absence of the GST-Syn(96-140) (Fig. 7). The thermal inactivation curves were used to determine the T_{50} values, the tempera-

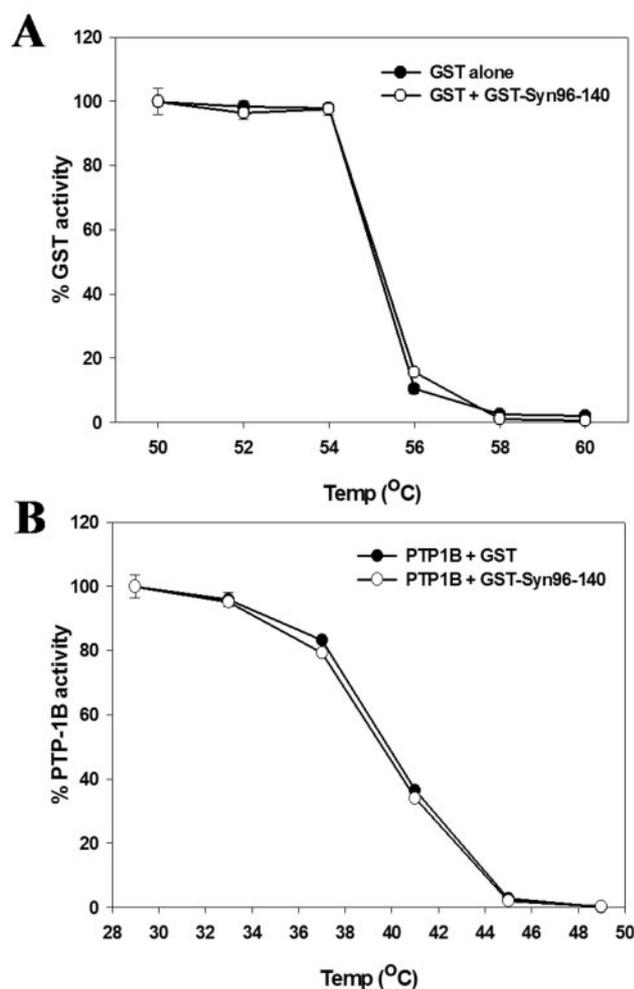
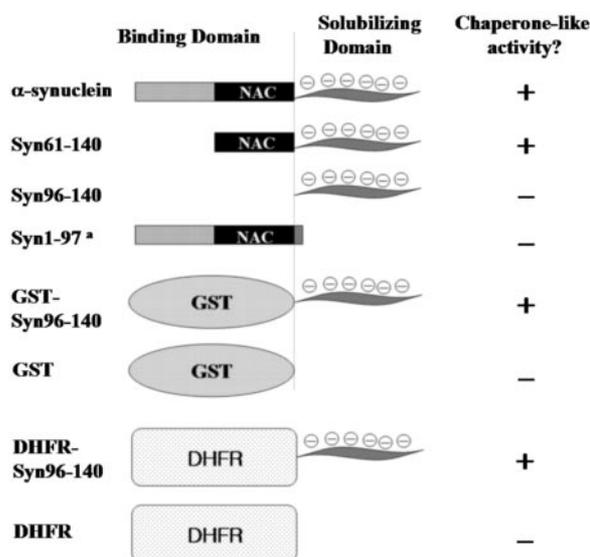


FIG. 7. Thermal inactivation curves of GST (A) and protein-tyrosine phosphatase-1B (B) in the presence and absence of GST-Syn(96-140). Activity is expressed as a percentage of initial activity. Values are the means of three independent experiments, and the standard deviation is shown as bars. The protein samples were incubated for 5 min at the indicated temperatures, and the enzymatic activities were measured as described under "Experimental Procedures" at 37 °C.

tures at which 50% of initial enzymatic activity was lost after heat treatment. As shown in Fig. 7, T_{50} values of GST and protein-tyrosine phosphatase-1B appeared to be similar in the presence and absence of the chaperone protein. This suggests that GST-Syn(96-140) was incapable of protecting enzymes from thermal inactivation, although it can prevent the enzymes from thermal aggregation.

DISCUSSION

α -Synuclein has been suggested to work as a chaperone protein in mammalian cells (18), and recent studies have shown that α -synuclein acts as a chaperone *in vitro*, and that the C-terminal truncated form of α -synuclein (Syn-(1-97)) has no chaperone activity (24, 25). In this study, we have shown that the C-terminal acidic tail itself (Syn-(96-140)) does not interact with the substrate protein and consequently does not protect the protein from stress-induced aggregation. However, in common with wild type α -synuclein, an N-terminal truncated form of α -synuclein (Syn-(61-140)) binds the substrate protein and retains the chaperone activity, albeit with a slightly different efficiency and substrate specificity. This indicates that the N-terminal region of α -synuclein (residues 1-95) plays a critical role in substrate protein binding, and that the



a. taken from ref 24.

FIG. 8. Distinct roles of the N-terminal-binding domain and the C-terminal-solubilizing domain of α -synuclein in its molecular chaperone function. Proteins containing both the binding domain and the solubilizing domain exhibit chaperone-like activity.

C-terminal acidic tail might function to solubilize the HMW complex (Fig. 8). Interestingly, the N-terminal-binding domain can be substituted by other proteins or peptides, such as GST, DHFR, or NAC peptide (Fig. 8), and the resulting fusion proteins were also found to have chaperone activity. More importantly, the synthetic chaperone proteins appear to display differential chaperone activity in terms of their efficiencies and substrate specificities (Figs. 1, 3, and 6).

The N-terminal part of α -synuclein (residues 1-61) is homologous to the chaperone protein 14-3-3 (18). Consequently, α -synuclein and 14-3-3 bind to many of the same proteins, such as PKC, BAD, ERK, and tyrosine hydroxylase (18, 22). α -Synuclein was also able to bind to 14-3-3, tau protein, tubulin, synphilin-1, and phospholipase D₂ with high specificity and affinity (16-21). Furthermore, expression of α -synuclein was greatly increased in 293 HEK cells under stress conditions (18). Based on these observations, it has been suggested that α -synuclein functions as a chaperone protein *in vivo* (18). This hypothesis has been supported by the *in vitro* chaperone activity of α -synuclein reported recently (24, 25). However, α -synuclein appears to be a much weaker chaperone than HSP27 in preventing thermally induced aggregation of alcohol dehydrogenase and chemically induced aggregation of insulin *in vitro* (24). Our data also indicate that α -synuclein can protect target proteins from stress-induced aggregation only at a sub-stoichiometric ratio (Fig. 1). Taken together, it seems likely that α -synuclein at least acts as a chaperone *in vivo* for specific target proteins, like the case of the 14-3-3 chaperone. Potential target proteins may include those proteins that are known to associate with α -synuclein.

Most chaperone proteins have a flexible, hydrophilic tail that is important for proper chaperone function. For example, GroEL is known to have flexible N- and C-terminal tails, which protrude into the central cavity of the molecule (66). SecB, a bacterial chaperone involved in protein export, has been proposed to have a highly flexible C-terminal region that is involved in binding to non-native proteins (67). X-ray crystallographic and NMR spectroscopic analyses have shown that sHSPs, such as HSP25, HSP16, and α -crystallin, contain a flexible C-terminal extension (27-29, 68). Other small molecu-

lar chaperones, such as clusterin and tubulin, are also known to have a flexible tail at the C terminus (65, 69). These flexible, hydrophilic tails have been suggested to play a critical role in substrate and chaperone protein interactions, and to function as a solubilizer (26, 53–56). In fact, a mutation in the C-terminal end or a deletion of the C-terminal end caused a significant decrease in the chaperone activity of α -crystallin (55, 57). Tubulin also lost its chaperone-like activity when the C-terminal acidic tail was removed by protease digestion (69). A previous study showed that the removal of the C-terminal acidic tail of α -synuclein abolished its chaperone activity (24). Our present data indicate that the C-terminal acidic tail was indeed necessary, but not sufficient for the chaperone function of α -synuclein. The acidic tail itself does not have chaperone activity, and does not appear to interact with the substrate protein. It is highly likely that the role of the introduced acidic tail is to increase protein solubility by electrostatic repulsions. It is well documented that the solubility of a protein is approximately proportional to the square of the net charge on the protein (70). In fact, introducing the acidic tail greatly decreases the pI and hydropathy values of the fusion protein (59), and the C-terminal truncated α -synuclein mutants are found to aggregate faster than the full-length α -synuclein under the same conditions (61–64). Furthermore, fusion proteins containing the α -synuclein acidic tail (GST-Syn-(96–140) and DHFR-Syn-(96–140)) has chaperone-like activity, which suggests that a possible role of the acidic tail in chaperone function might involve solubilizing the substrate-chaperone complex, as well as the chaperone protein itself.

We have shown that the N-terminal region of α -synuclein binds the substrate protein forming a soluble HMW complex (Fig. 2). The GST-Syn-(96–140) fusion protein also appears to form such a HMW complex, suggesting that the GST domain interacts with the substrate protein (Fig. 4). Interestingly, however, the efficiency of the chaperone function of GST-Syn-(96–140) differs from that of wild type α -synuclein. GST-Syn-(96–140) appeared to be more efficient than α -synuclein at preventing GST and aldolase from heat-induced aggregation, but less efficient at preventing DTT-induced aggregation of insulin and lysozyme (Fig. 3). In addition, an N-terminal truncated form of α -synuclein (Syn-(61–140)) appeared to be more efficient than wild type α -synuclein at preventing proteins from DTT-induced aggregation (Fig. 1). These results strongly suggest that the N-terminal-binding domain plays a crucial role in the efficiency of the chaperone function. This idea is further supported by the observation that GST-Syn-(96–140) effectively prevents insulin from DTT-induced aggregation at elevated temperatures (Fig. 6A). At the elevated temperatures, the tertiary structure of GST must be changed and the perturbed structure seems to become more favorable for substrate protein binding. Furthermore, GST-Syn-(96–140) does not protect luciferase from heat-induced aggregation (Fig. 6B), although it effectively protects GST and aldolase (Fig. 3, A and B) and α -synuclein was able to protect all these molecules from heat-induced aggregation (Figs. 1, A and B, and 6B). These results suggest that the chaperone function of GST-Syn-(96–140) was much more specific/limited than that of α -synuclein. Taken together, our data demonstrate that the N-terminal-binding domain governs the efficiency and the substrate specificity of the chaperone proteins.

The chaperone action of sHSPs requires a common step of substrate protein binding and a subsequent step of solubilizing the HMW complex of chaperone and substrate protein (27, 32–40). The present study demonstrates that α -synuclein functions in the same manner as the sHSPs; α -synuclein prevents protein aggregation by binding substrate protein and subse-

quently by solubilizing the HMW complex. Furthermore, our results show that the substrate-binding domain and the solubilizing domain are clearly separated in α -synuclein; the N-terminal region (residues 1–95) binds the substrate protein and the C-terminal acidic tail (residues 96–140) solubilizes the HMW complex. Unlike α -synuclein, however, sHSPs do not appear to have well separated substrate-binding and -solubilizing domains. In the case of sHSPs, the hydrophobic and the charged/hydrophilic regions are scattered through the N- and C-terminal domains (49). sHSPs also have short, hydrophilic extensions at the C terminus (10–15 residues), but these C-terminal extensions play a significant role in substrate binding, as well as in solubilizing the HMW complexes (54, 56). Furthermore, C-terminal truncated forms of sHSPs still retain the chaperone activity, although the chaperone activity is somewhat reduced and limited in some cases (38, 51, 55, 57). This further suggests that the amino acid residues responsible for substrate binding and solubilizing sHSP-based HMW complexes are scattered through the whole of the sHSP molecules.

The chaperone activity of sHSPs toward DTT- and UV-induced protein aggregation is enhanced as the temperature increases, because the conformation of sHSPs is presumably perturbed and consequently hydrophobic surfaces are more exposed at the higher temperatures (40, 71–76). A similar temperature-dependent interaction between GroEL and substrate protein has been reported (77). The chaperone-like activity of tubulin also becomes more pronounced as temperature increases (69). Previously, we reported that preheating α -synuclein, which is believed to reorganize the molecular surface of the protein, increases its chaperone activity (25). In this study, we demonstrated that GST-Syn-(96–140) more efficiently protects insulin from DTT-induced aggregation at 59 °C than at room temperature (Fig. 6A). Therefore, as has been observed for other molecular chaperone proteins, temperature-induced structural perturbation of the GST domain (substrate-binding domain) seems to be responsible for the increased chaperone-like activity observed at higher temperatures.

The list of the new small molecular chaperones discovered is increasing quite steadily, and recently tubulin, clusterin, and nucleolar protein B23 have been added as new members (65, 69, 78). Tubulin also has a C-terminal acidic tail and the removal of this tail abolishes its chaperone-like activity (69). Clusterin contains three putative amphipathic α -helical regions that might mediate interaction with hydrophobic molecules (65), and nucleolar protein B23 has been reported to have chaperone-like activity through a similar mechanism (78). These chaperone proteins (tubulin, clusterin, and B23) have no amino acid sequence similarity with either sHSPs or α -synuclein. Therefore, it would be interesting to compare detailed molecular mechanisms of the chaperone action mediated by these molecules with those of α -synuclein and sHSPs.

In summary, the results of this study indicate that the N-terminal region of α -synuclein binds substrate protein and forms a HMW complex, whereas the C-terminal acidic tail solubilizes the HMW complex during the chaperone action. Because the substrate-binding domain and the solubilizing domain are well separated in α -synuclein, the N-terminal-binding domain can be substituted with other proteins or peptides. Moreover, the resulting engineered chaperone proteins appear to display different efficiencies and substrate specificities in terms of the chaperone function. This implies that the C-terminal acidic tail of α -synuclein can be utilized to engineer synthetic chaperones for specific purposes simply by fusing the acidic tail with other proteins or peptides. Such specifically designed chaperone proteins would be useful for stabilizing target proteins both *in vitro* and *in vivo*.

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D. Kim, Jeon Han Park, Chul-Hak Yang and
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