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Structural changes in α-synuclein affect its chaperone-like activity in vitro

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Abstract

α-Synuclein, a major constituent of Lewy bodies (LBs) in Parkinson's disease (PD), has been implicated to play a critical role in synaptic events, such as neuronal plasticity during development, learning, and degeneration under pathological conditions, although the physiological function of α-synuclein has not yet been established. We here present biochemical evidence that recombinant α-synuclein has a chaperone-like function against thermal and chemical stress in vitro. In our experiments, α-synuclein protected glutathione S-transferase (GST) and aldolase from heat-induced precipitation, and α-lactalbumin and bovine serum albumin from dithiothreitol (DTT)-induced precipitation like other molecular chaperones. Moreover, preheating of α-synuclein, which is believed to reorganize the molecular surface of α-synuclein, increased the chaperone-like activity. Interestingly, in organic solvents, which promotes the formation of secondary structure, α-synuclein aggregated more easily than in its native condition, which eventually might abrogate the chaperone-like function of the protein. In addition, α-synuclein was also rapidly and significantly precipitated by heat in the presence of Zn^{2+} in vitro, whereas it was not affected by the presence of Ca^{2+} or Mg^{2+}. Circular dichroism spectra confirmed that α-synuclein underwent conformational change in the presence of Zn^{2+}. Taken together, our data suggest that α-synuclein could act as a molecular chaperone, and that the conformational change of the α-synuclein could explain the aggregation kinetics of α-synuclein, which may be related to the abolishment of the chaperonic-like activity.

Keywords: α-synuclein; molecular chaperone; Parkinson's disease; protein folding; structural change

Parkinson's disease (PD), a neurodegenerative disorder associated with dopaminergic nerve cell loss, is characterized by the presence of neuronal inclusion bodies and dystrophic neurites predominantly in the substantia nigra. A pathological hallmark of PD is the presence of intracytoplasmic Lewy bodies (LBs), which also accumulated in brains with dementia and multiple system atrophy. The major filamentous component of Lewy bodies is α-synuclein, a presynaptic protein of the central nervous system (Heintz & Zoghbi, 1997; Baba et al., 1998; Clayton & George, 1998). The α-synuclein immunoreactivity to Lewy bodies and Lewy neurites in patients with sporadic PD and dementia with LBs is already known, although LBs have been shown to be immunoreactive also for beta-amyloid precursor protein and ubiquitin C-terminal hydrolase (Leroy et al., 1998; Spillantini et al., 1998). Moreover, transgenic mice overexpressing α-synuclein develop inclusion bodies and a loss of dopaminergic terminals, supporting the idea that abnormal aggregation of α-synuclein might play a central role in PD (Masliah et al., 2000).

α-Synuclein comprises 140 amino acids in two domains, linked via the NAC sequence (Fig. 1A). The C-terminal domain is rather acidic. The N-terminal domain, which is highly conserved between species, comprises seven repeats of 11-amino-acid motif (residues 9–89). Fast axonal transport of vesicle-bound α-synuclein is dependent on the integrity of the first N-terminal four of the 11-amino-acid repeats (Jensen et al., 1998). α-Synuclein is unfolded in its native state, perhaps explaining its ability to interact with many other proteins or ligands (Weinreb et al., 1996; Kim, 1997). Interestingly, α-synuclein, with very little secondary structure in aqueous solution, associates with small acidic phospholipid vesicles and acquires increased level of secondary structure (Davidson et al., 1998).

The importance of α-synuclein in human neurodegenerative disease was first proposed when a 35-amino-acid peptide correspond-

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Abbreviations: CD, circular dichroism; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PD, Parkinson's disease; AD, Alzheimer disease; HD, Huntington disease; LB, Lewy bodies; GST, glutathione S-transferase; DTT, dithiothreitol; BSA, bovine serum albumin; HSP, heat-shock protein; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl-βD-thiogalactoside; PMSF, phenylmethylsulfonyl fluoride.

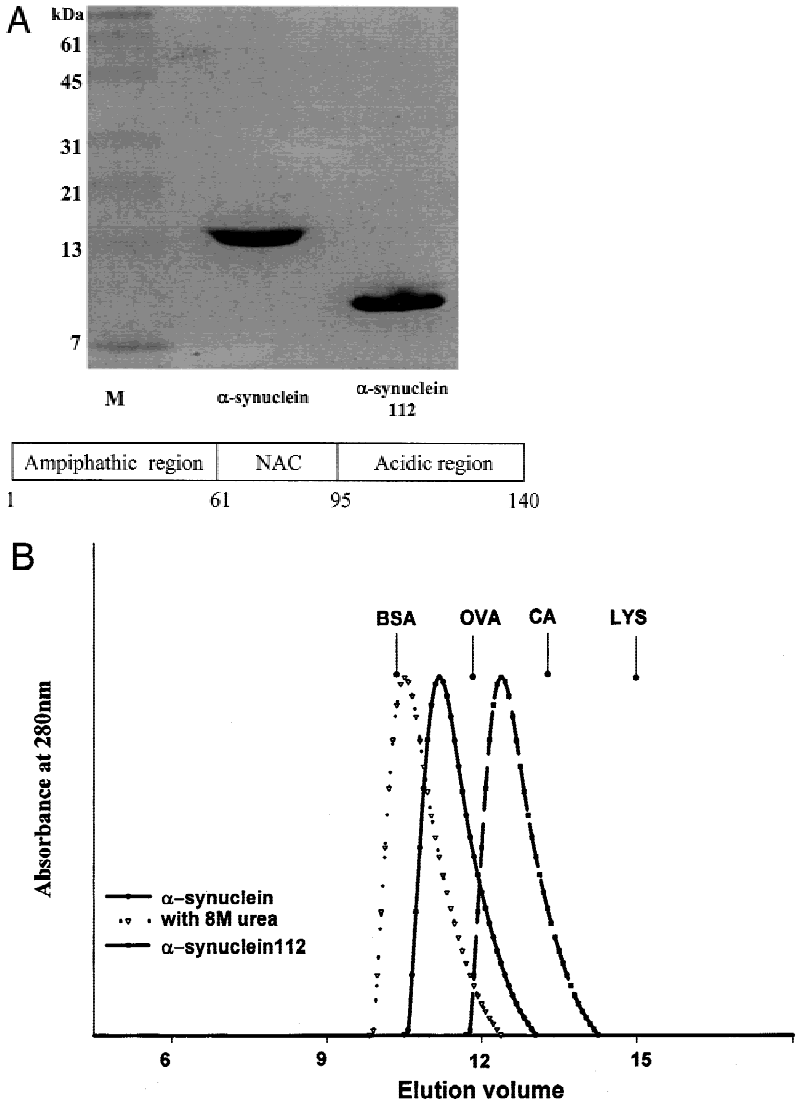

Fig. 1. A: Purified proteins of α-synuclein and α-synuclein112 were separated on a 15% SDS polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue R-250. Lane 1, α-synuclein (14.4 kDa); lane 2, synuclein112 (11.4 kDa). Molecular weights of the size markers are shown at the left. Three distinct regions of α-synuclein designated amphipathic region (N-terminus region), NAC, and acidic region (C-terminal regions) are shown in the bottom of the figure. **B:** Schematic elution profiles and elution volumes (Ve) of α-synuclein and α-synuclein112 in size-exclusion chromatography were shown under various conditions; α-synuclein in MES buffer (10.84 mL), in 8 M urea (9.86 mL), and α-synuclein112 (12.08 mL). Elution volumes of molecular weight standard proteins are also marked above the figure (BSA, ovalbumin, carbonic anhydrase, and lysozyme).

ing to residues 61–95 in α-synuclein was purified from Alzheimer's disease senile plaques (Ueda et al., 1993). Although the mechanism by which α-synuclein is intracellularly aggregated during neurodegeneration in PD or related disorders is not yet fully understood, it has been reported that α-synuclein, as a whole or a partially truncated molecule, was shown to be aggregated to form amyloid-like fibrils that contain high percentages of beta-pleated sheet structure (Takeda et al., 1998; Serpell et al., 2000). Two different point mutations (A53T and A30P) in α-synuclein gene on human chromosome 4 have been identified with early onset inherited form of PD (Polymeropoulos et al., 1997; Kruger et al., 1998). These mutations accelerate the aggregate formation in vitro, which may explain the aggregation kinetics of α-synuclein in the pathogenesis of PD (Conway et al., 1998). It has also been shown that the in vitro aggregation of α-synuclein is modulated by various factors such as Aβ, cytochrome *c*, hydrogen peroxide, SDS, and metal ions (Paik et al., 1998, 1999; Hashimoto et al., 1999).

Structural features and the conformational change of α-synuclein led us to the hypothesis that α-synuclein may bind to hydrophobic regions of partly unfolded proteins by stresses and act as a molecular chaperone. In addition, we hypothesized that perturbation of α-synuclein structure by organic solvent, metal ions, and heat could affect the chaperonic activity and aggregation kinetics of α-synuclein. In this study, we showed that (1) α-synuclein can act as a molecular chaperone in its native state in vitro, and (2) conformational change by organic solvent or zinc ion induces the aggregation of α-synuclein, while heat-incubated α-synuclein enhances the chaperone-like function. These results suggest that struc-

tural change of α-synuclein might explain the aggregation property and loss of chaperone activity of α-synuclein, which might be related to the formation of Lewy body fibrils in Parkinson's disease.

Results

Gel filtration chromatography

Figure 1A shows the SDS-PAGE analysis of α-synuclein and α-synuclein112 proteins. The recombinant proteins were purified ~95% homogenity as evident from the Coomassie blue-stained SDS-PAGE gel. To characterize hydrodynamic properties of α-synuclein and its deletion mutant form at neutral pH, gel-filtration chromatography was performed. The protein was loaded onto the gel-filtration column and eluted as a single sharp peak corresponding to relative molecular mass of 60 kDa for α-synuclein and 42 kDa for α-synuclein112 compared to molecular-size markers (Fig. 1B). The sizes of α-synuclein and α-synuclein112 were observed to be larger than the one predicted from amino acid sequences (14.4 and 11.4 kDa, respectively). Our data are in agreement with the previous reports that α-synuclein is predominantly monomeric and unstructured with large Stokes radius for its monomeric state (Kim, 1997; Conway et al., 2000). Deletion of acidic tail region in α-synuclein112 did not cause the compaction of its unstructured nature in gel-filtration. Interestingly, α-synuclein seems to be more unfolded in the presence of 8 M urea with larger stokes radius than in physiological buffer solution, which implies some tertiary interactions existing in α-synuclein are disrupted under this condition. Deletion of 43 amino acids in C-terminus region did not lead to an alteration in the unstructured nature of synuclein in gel filtration chromatography and circular dichroism spectra (data not shown).

CD spectroscopy

To examine the effect of temperature and organic solvent on the secondary structure of α-synuclein, far-UV CD spectra were investigated (Fig. 2). The far-UV CD spectra of α-synuclein had a characteristic minimum around 200 nm, which is indicative of high proportion of random coil as much as 70%. At 100 °C, α-synuclein showed a reduced minimum around 200 nm. The CD spectrum of preincubated α-synuclein reflected a possibility of structural reorganization. We have also investigated the effect of organic solvent, hexafluoroisopropanol (HFIP), which is shown to induce secondary structure. As expected, the far-UV CD spectra of α-synuclein in this organic solvent showed ordered secondary structure with α-helix and β-sheets. Interestingly, as shown in Figure 3, thermal stress in 10% HFIP condition causes dramatic increase of aggregation in both cases of α-synuclein and α-synuclein 114. Therefore, it is evident that the heat-induced aggregation level is greatly increased with the conformational change of α-synuclein induced by HFIP.

Effects of metal ions

We have investigated the aggregation level of α-synuclein in the presence of various metal ions (Fig. 4A). α-Synuclein was incubated with different divalent metal ions at 20 mM MES (pH 6.5), and turbidity was measured at 360 nm. Mg^{2+} and Ca^{2+} were observed to induce no significant aggregation. Only incubation

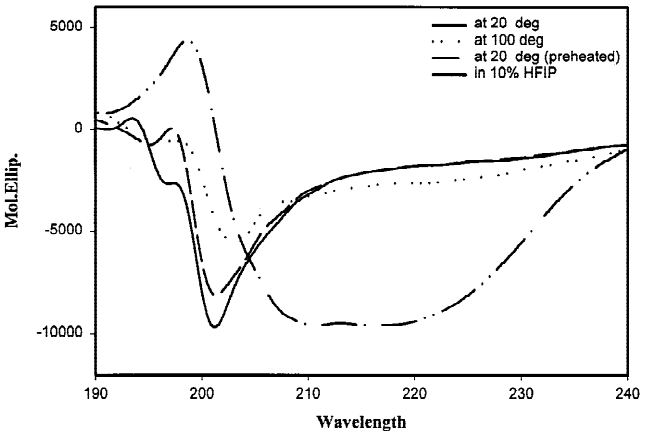

Fig. 2. Far-UV CD spectra of α-synuclein under various conditions. CD spectra of α-synuclein at 20 °C (solid line), 100 °C (dotted line), and 20 °C (preincubated, dashed line) are shown, respectively. CD spectra of α-synuclein in the presence of 10% HFIP at 20 °C are also shown in solid-dotted line. Note that the negative minima around 200 nm of random coil region is changed by temperature and organic solvent.

with Zn^{2+} induced significant aggregation under these conditions. The light-scattering data for α-synuclein indicated that there is no aggregation in this concentration (10 μM), but the addition of Zn^{2+} appeared to result in aggregation like the case of GroEL (Brazil et al., 1998). Interestingly, Zn^{2+}-induced turbidity was abolished by the presence of the EDTA, metal ion chelator. The protein BSA (0.1 mg/mL) did not aggregate in the presence of Zn^{2+} (data not shown), which suggestes that this effect of Zn^{2+} on α-synuclein reflects a specific interaction. Doubling the concentration of α-synuclein with Zn^{2+} increased the aggregation level (Fig. 4B). In addition, Zn^{2+} caused structural reorganization of the

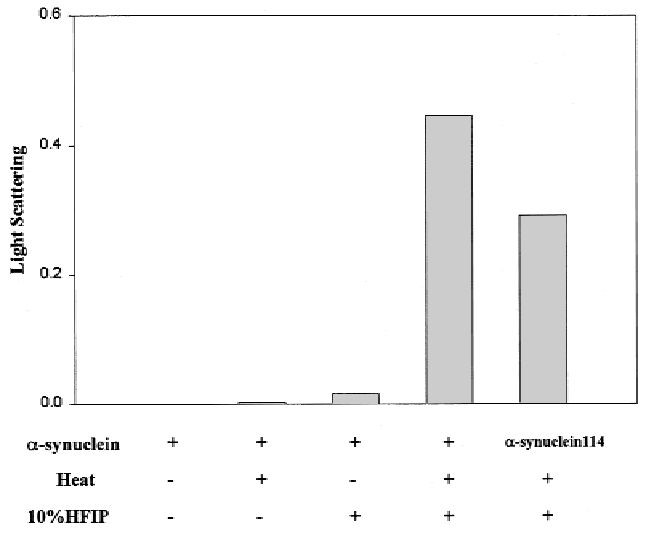

Fig. 3. Thermal aggregation of α-synuclein was increased significantly in the presence of organic solvent (10% HFIP). α-Synuclein (10 μM) was aggregated more easily by heat when the organic solvent induces the secondary structure of α-synuclein. α-Synuclein114 was also aggregated in the presence of heat and organic solvents.

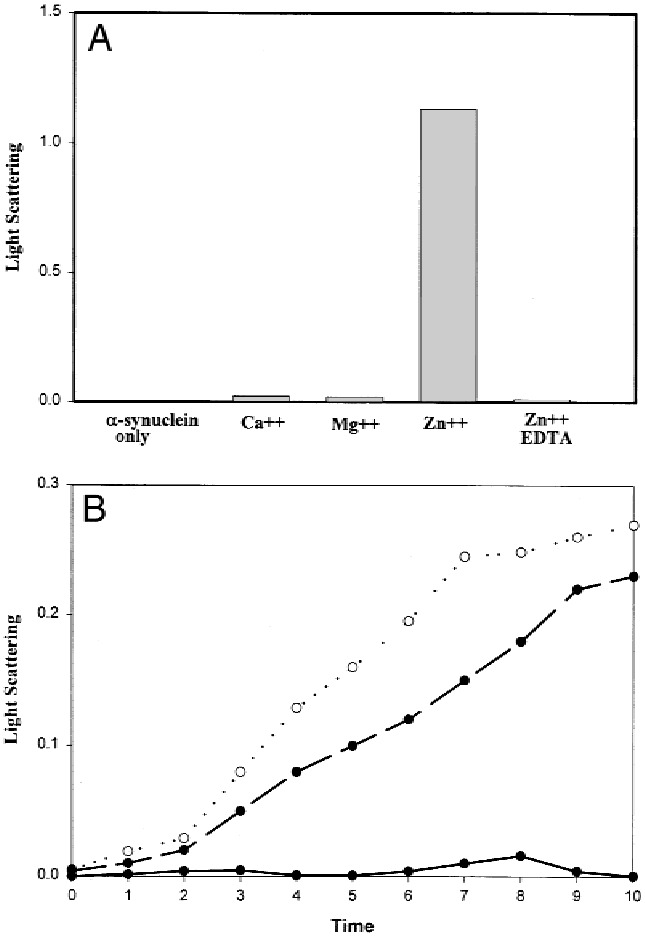

Fig. 4. **A:** Turbidometric analysis of metal induced α-synuclein aggregation. α-Synuclein were incubated with each metal ion (100 μM). EDTA (250 μM) was added for chelation of Zn^{2+}. All samples were heated at 65 °C for 10 min. **B:** Light scattering of α-synuclein were investigated in the presence of zinc; α-synuclein 20 μM (open circle, dotted) or 10 μM (filled circle, dashed line) were heated at 65 °C in the presence of 50 μM zinc. α-Synuclein without zinc (filled circle, solid line) was also heated at 65 °C.

α-synuclein in CD spectra (data not shown). Therefore, Zn^{2+} could cause conformational change of α-synuclein structure and leads to the increase of aggregation like the case of organic solvent. This finding is reminiscent of the behavior of Aβ, which showed increased aggregation through conformational change by organic solvents and Zn^{2+} (Huang et al., 1997).

Chaperone-like activity of α-synuclein

We have investigated that α-synuclein could prevent the aggregation of a variety of unrelated proteins/enzymes caused by thermal and chemical stress like small heat-shock proteins. Suppression of the aggregation of nonnative conformations of substrate proteins by α-synuclein is used to measure its chaperone-like activity in vitro. The chaperone-like activity of α-synuclein against heat- and DTT-induced aggregation was investigated by the apparent absorbance of light scattering. Figure 5 shows the kinetic traces of the apparent light scattering of aldolase and glutathione S-transferase (GST) in the presence and absence of α-synuclein against heat treatment. When solutions of substrate proteins, such as aldolase and GST, were heated at 65 °C for 10 min, the solutions got turbid because of the formation of large insoluble aggregates (Fig. 5A,B, curve 1). However, α-synuclein alone did not precipitate when heated at 65 °C for 30 min or treated with DTT. Comparison of curves 1 and 2 in Figure 5A indicates the capability of α-synuclein to suppress the aggregation of aldolase. In the presence of α-synuclein (1:1 w/w aldolase:α-synuclein), aggregation was suppressed to as much as 50%. Doubling the concentration of α-synuclein increased the suppression of the aggregation. In Figure 5B, GST underwent extensive aggregation within 5 min at 65 °C, shown by an increase in absorbance at 360 nm. However, in the presence of α-synuclein (1:1 w/w GST: α-synuclein), aggregation was suppressed to as much as 60%. Increasing the concentration of α-synuclein suppresses the aggregation of GST like in the case of aldolase. Interestingly, α-synuclein could supress the aggregation of GST more efficiently, which might be resulted from the small molecular weight of GST (29 kDa) compared to aldolase (150 kDa). In addition, bovine se-

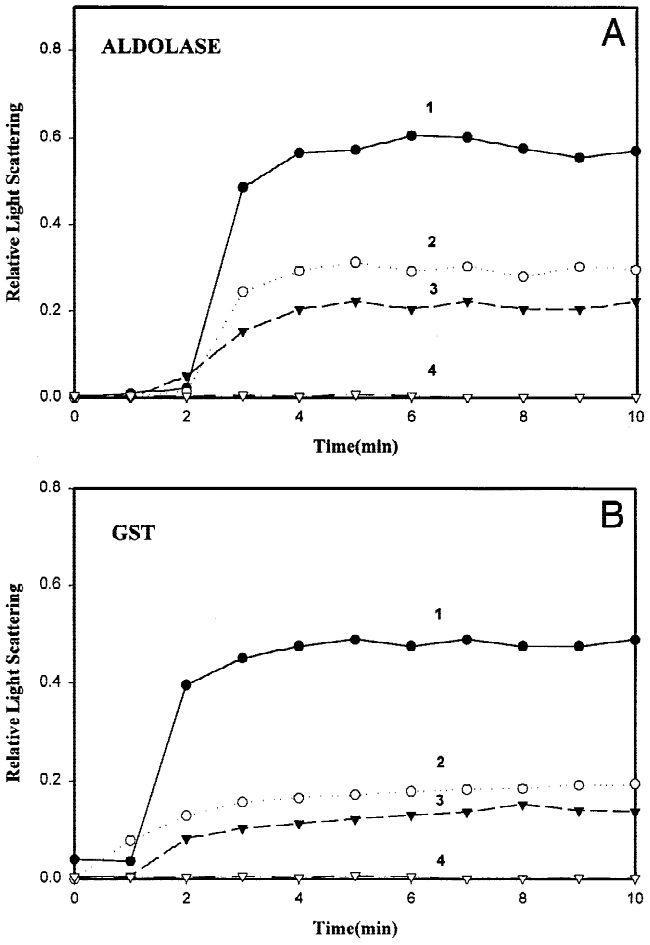

Fig. 5. α-Synuclein suppress the thermal aggregation of aldolase and GST. **A:** Aggregation curves of aldolase (0.2 mg/mL) in the absence of α-synuclein (filled circle), aldolase/α-synuclein (1:1 w/w, open circle), aldolase/α-synuclein (1:3 w/w, filled triangle), and α-synuclein only (0.2 mg/mL, open triangle). **B:** Aggregation curves of GST (0.2 mg/mL) in the absence of α-synuclein (filled circle), GST/α-synuclein (1:1 w/w, open circle), GST/α-synuclein (1:3 w/w, filled triangle), and α-synuclein only (0.2 mg/mL, open triangle).

rum albumin (BSA) or lysozyme did not protect GST or aldolase against thermal aggregation regardless of the amount of proteins used (data not shown).

Analysis of reaction mixtures by size-exclusion chromatography indicated the formation of high molecular weight species with a larger Stokes radius, which are composed of α-synuclein and substrate protein (data not shown). These results indicates that α-synuclein binds to stressed proteins with the formation of high molecular weight complexes like the other molecular chaperones (Rao et al., 1993; Rajaraman et al., 1996).

The kinetics of aggregation by disulfide bonds reduction were monitored by measuring the apparent absorbance of light scattering at 360 nm (Horwitz et al., 1998). Figure 6A shows that α-synuclein suppressed DTT-induced aggregation of BSA in a concentration-dependent manner (curves 2 and 3). α-Synuclein also offered comparable protection against DTT-induced aggregation of bovine α-lactalbumin (Fig. 6B, curves 1 and 2). It is evident from these experiments that α-synuclein suppresses the DTT-induced as well as heat-induced precipitation. Interestingly, preheated α-synuclein suppressed the aggregation process more effectively compared to the nonheated one. For lysozyme as a substrate, only preheated α-synuclein suppressed the aggregation (Fig. 6C). Structural reorganization observed in CD spectra (Fig. 2) could be related to this enhanced molecular chaperone-like activity. This finding is consistent with the behavior of other known chaperones such as α-crystallin and tubulin, whose activities are generally found to increase with the gain of temperature (Datta & Rao, 1999; Haslbeck et al., 1999). It is also known that a slight perturbation of its conformation by heat or chaotropic agents results in an increase in its hydrophobicity or reorganization of the surfaces and, therefore, its substrate binding capacity (Lindner et al., 1998).

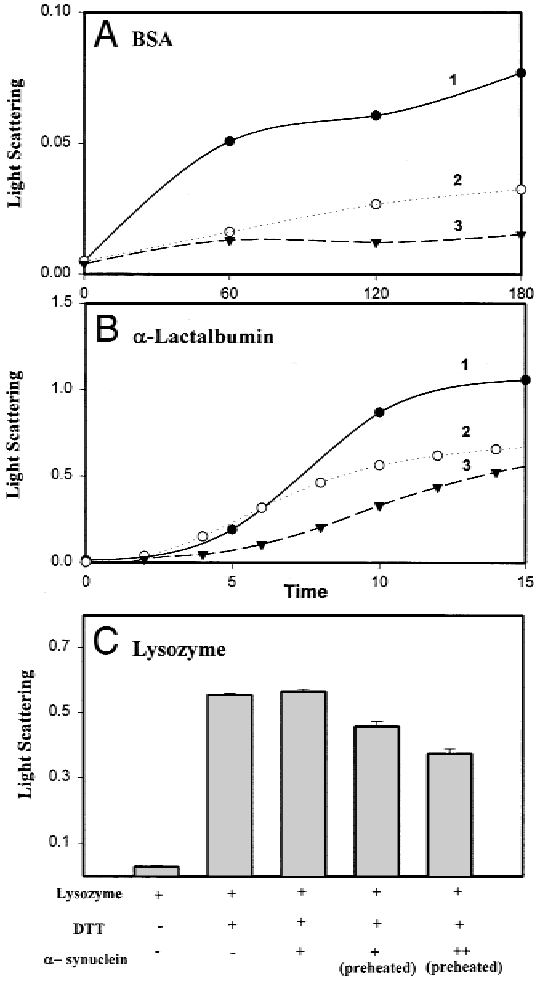

Fig. 6. DTT-induced aggregation kinetics of BSA, bovine α-lactalbumin, and lysozyme in the presence and absence of α-synuclein. All samples were treated with 20 mM DTT at 37 °C. (**A**) Aggregation curves of BSA in the absence of α-synuclein (curve 1), BSA/α-synuclein (1:0.25 w/w, curve 2), and BSA/α-synuclein (1:0.5 w/w, curve 3). (**B**) Aggregation curves of α-lactalbumin (0.5 mg/mL) in the absence of α-synuclein (curve 1), α-lactalbumin/α-synuclein (1:0.6 w/w, curve 2), and α-lactalbumin/preincubated α-synuclein (1:0.6 w/w, curve 3). For preincubation, α-synuclein was preheated at 65 °C for 10 min and cooled to room temperature before the measurement. (**C**) Aggregation curves of lysozyme (0.5 mg/mL) in the absence and presence of α-synuclein lysozyme/α-synuclein (w/w) is 1:1 except the last column (1:2). For preincubation, α-synuclein was preheated at 65 °C for 10 min and cooled to room temperature.

α-Synuclein does not protect enzyme from heat-induced loss of activity

To investigate whether α-synuclein is also capable of protecting enzymes from stress-induced loss of activity, we tested the enzyme activity of GST before and after exposure to heat in the presence or absence of α-synuclein (Fig. 7). After the exposure of GST at 65 °C for 10 min, the enzyme completely lost its activity. Interestingly, α-synuclein had a negligible effect on the heat-induced loss of function of GST, although addition of α-synuclein suppresses the aggregation. Therefore, α-synuclein could not protect enzymes from the loss of its activity, although it has chaperone-like properties of protecting stressed proteins from precipitation. Previous studies have shown that HSP25, clusterin, and α-crystallin also protect proteins from heat-induced precipitation, but only α-crystallin is capable of protecting against enzyme inactivation (Carver et al., 1994; Humphreys et al., 1999).

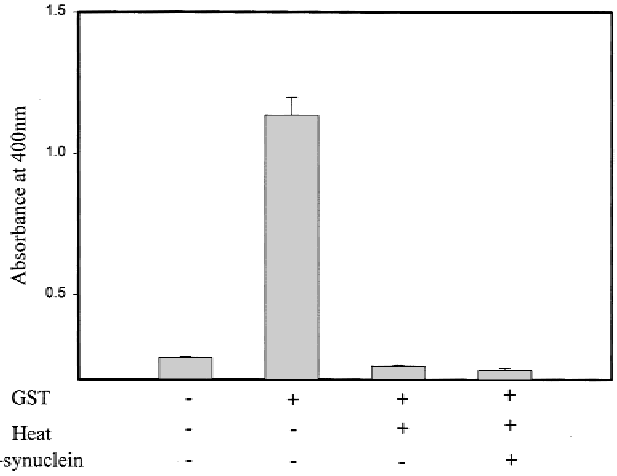

Fig. 7. Enzyme activity of GST following incubation at 65 °C for 10 min. GST with or without α-synuclein was heated, and enzyme activity was measured as an increase in absorbance at 350 nm. Each histogram represents the mean of three independent measurements, and the error bars represent standard errors.

Discussion

In many neurodegenerative diseases, filamentous lesions are observed in the extracellular, intracytoplasmic, or nuclear regions. Each lesion is mainly composed of insoluble fibrils of proteins such as α-synuclein in PD, Aβ in AD amyloid plaque, tau in nneurofibrillary tangles, and huntington in HD (Kaytor & Warren, 1999; Trojanowski & Lee, 1999). An increasing number of evidence suggests that filamentous aggregates resulting from abnormal protein–protein interactions play a mechanistic role in the dysfunction and death of neurons and/or glia in many neurodegenerative diseases (Goedert et al., 1998; Wolozin & Behl, 2000). Although filamentous α-synuclein aggregates in PD play a significant role in the pathogenesis of neurodegenerative disorders, the mechanism of fibrillogenesis and function of α-synuclein have not yet been fully addressed. We present here biochemical evidence suggesting that α-synuclein have a chaperone-like function for proteins denatured by thermal and chemical stress. This function is regulated by environmental factors (temperature, metal, organic solvent), which could induce the structural reorganization of α-synuclein. Therefore, structural transitions of α-synuclein, which might be a key toward the understanding of the aggregation of α-synuclein, are related to the chaperone-like function of α-synuclein *in vitro*.

All molecular chaperones require a common step of binding the partially or completely unfolded substrate and thereby preventing the off-pathway reaction that leads to protein aggregation. A common feature of partly folded and unfolded polypeptides induced by heat or disulfide bond reduction is the solvent exposure of hydrophobic residues that are buried in its native structure. Several studies of molecular chaperones have revealed the importance of hydrophobic residues in binding of substrate polypeptides (Ehrnsperger et al., 1997; Dobson & Karplus, 1999). Our data indicate that α-synuclein effectively protects GST and aldolase from heat-induced precipitation, and bovine α-lactalbumin, BSA, and lysozyme from DTT-induced precipitation. These observation suggest that α-synuclein has chaperone-like properties that could prevent the aggregation of a number proteins that are unrelated in structure or sequence, as has been recently reported by Souza et al. (2000). Size-exclusion chromatography showed the formation of high molecular weight complex. Therefore, it is tempting to think that α-synuclein binds to exposed hydrophobic regions on stressed proteins to form high molecular weight complexes and the formation of these complexes prevents the precipitation of the substrate proteins.

Conformational transitions of α-synuclein may be closely linked to the chaperone-like activity and aggregation of α-synuclein in PD. As shown in Figure 1, size-exclusion chromatography reveals that α-synuclein has some ordered structure that could be disrupted in 8 M urea condition, although α-synuclein has little secondary structure in a buffer condition. Interestingly, in organic solvent or in the presence of Zn^{2+}, α-synuclein seems to have more ordered structure than its native state. In a 10% HFIP condition, which promotes the formation of α-helix, the molecular surface of α-synuclein changes and leads to aggregation easily by heat, which could be interpreted as the abolishment of the chaperone-like function. Moreover, α-synuclein is rapidly and significantly precipitated by Zn^{2+}, which could also change the secondary structure of α-synuclein. In this respect, it is possible that Zn^{2+} act by shielding the negative charge on α-synuclein, and consequently, abrogates the chaperone-like activity. It has also been reported that interaction of α-synuclein with Cu^{2+}/Fe^{3+} results in the overproduction of free radicals that are associated with PD as a causal factor in the death of cells (Hashimoto et al., 1999; Paik et al., 1999). Therefore, organic solvent and metal ions appears to act in a more general fashion by modulating the hydrophobic surface of α-synuclein, which eventually leads to self-aggregation, and could abolish its binding to nonnative conformation of polypeptides. Preheating of α-synuclein is characterized by a partial structural change of secondary structure in the CD spectra (Fig. 2). The conformational transition induced by heating seems to be involved in enhancing the chaperone-like activity of α-synuclein. Additionally, Hsp90 and α-crystalline undergoes a temperature-dependent structural perturbation, which results in an increase in its chaperone-like activity (Surewicz & Olesen, 1995; Yonehara et al., 1996; Burgio et al., 2000). It has been suggested that these changes could be explained as the reorganization of the hydrophobic surfaces and/or small structural perturbation of these molecules (Raman & Rao, 1997; Chadli et al., 1999). Interestingly, α-synuclein was unable to protect heat-induced loss of activity of GST (Fig. 7). Consistent with this observation, HSP25, clusterin, and α-crystalline also protect proteins from thermally induced precipitation, but only α-crystalline is capable of protecting against enzyme inactivation (Horwitz, 1992; Hook & Harding, 1997; Humphreys et al., 1999). In this respect, it would be interesting to see in more detail if α-synuclein helps the recovery of other enzyme activity inactivated by heat treatment.

Like other chaperones, α-synuclein appears to prevent irreversible aggregation of proteins induced by thermal as well as nonthermal stress by providing molecular surfaces to the unfolding proteins. Demonstration of chaperone-like activity of α-synuclein has provided an opportunity to investigate the mechanistic and functional aspects of α-synuclein under stress conditions, with the possibility that α-synuclein may have a function of interacting with aged or damaged proteins. Our observation that α-synuclein can function as a molecular chaperone may be significant in understanding the mechanism of fibrillogenesis in PD, although it is not clear at present whether α-synuclein may assist the folding of other proteins in vivo. It has been known that overexpression of molecular chaperones such as sHSPs and clusterin protected cells from the cytotoxicity of tumor necrosis factor (Humphreys et al., 1997; Michel et al., 1997; Arrigo, 1998). Multiple lines of evidence have been accumulated that α-synuclein could act to protect cells from environmental stresses (da Costa et al., 2000; Kanda et al., 2000). Overexpression of α-synuclein protects neuronal cells from apoptotic stimuli and PD-related mutation and C-terminal truncation of α-synuclein enhance vulnerability to oxidative stress of hydrogen peroxide and MPTP. Therefore, chaperone-like activity of α-synuclein observed in vitro could function as a cell survival factor in vivo. This study shows that the chaperone-like activity of α-synuclein seem to be abolished upon structural perturbation or reorganization by Zn^{2+} and organic solvents, while preheating of the protein could enhance the chaperone-like activity in vitro. Therefore, α-synuclein seems to have molecular chaperone function, and the conformational change of α-synuclein may be a way of modulating the binding of unfolded polypeptide substrates.

Materials and methods

Materials

Bovine serum albumin (BSA), bovine α-lactalbumin, IPTG, glutathione, urea, and 1-chloro-2,4-dinitrobenzene were purchased from Sigma (St. Louis, Missouri). Aldolase was obtained from Amer-

sham Pharmacia Biotech (Uppsala, Sweden). Dithiothreitol (DTT) was obtained from Boehringer Mannheim (Mannheim, Germany). Reagents used for SDS-PAGE were from Bio-Rad (Hercules, California). All reagents used were of analytical grade. GST from *Schistosoma japonicum* was prepared by the expression of pGEX-4T in *Escherichia coli.* The recombinant protein was purified by GSH-agarose affinity chromatography, as the manufacturer suggested. The purities of the proteins were verified by SDS-PAGE, and protein concentrations were determined by using the Bio-Rad Bradford reagent (Hercules, California) or by absorbance measurement at 280 nm.

Methods

Protein preparation

The plasmids containing full-length α-synuclein and α-synuclein112 gene were generous gifts from Dr. R. Jakes (MRC, England) and Dr. W. Choi (Chongju University, Korea), respectively. α-Synuclein and α-synuclein114 was prepared as described previously (Paik et al., 1998). For α-synuclein112, which lacks the residues of 103–130 in the acidic tail, BL21(DE3)pLysS harboring synuclein112 gene were grown in LB medium at 37 °C to an A_{600} of 1.0 and then grown for another 4 h after induction with 0.5 mM IPTG. Cells were harvested by centrifugation at 7,000 × *g* for 10 min, resuspended in phosphate-buffered saline, and disrupted by sonication. After removing cell debris, the supernatants were subjected to heat treatment at 100 °C for 20 min and were dialyzed at 4 °C against 50 mM sodium acetate buffer, pH 4.6 for 16 h, with buffer exchange at 3 and 12 h. Then they were loaded on a Mono S-Sepharose column preequilibrated with 50 mM sodium acetate buffer (pH 4.6) and eluted with a linear gradient of 0–0.4 M NaCl in the same buffer. The proteins were concentrated using Centricon concentrators (Amicon, Beverly, Massachusetts) of molecular mass cutoff 10 kDa. The protein was further purified using Superdex75 gel-filtration column equilibrated with phosphate-buffered saline, pH 7.4. The protein was concentrated and stored at −20 °C before use. Protein samples were centrifuged at 12,000 × *g* for 5 min before all experiments and purities of the proteins were verified by SDS-PAGE (Fig. 1).

Size-exclusion chromatography

The Superdex 75 HR 10/30 column (Pharmacia, Uppsala, Sweden) was equilibrated with phosphate buffered-saline (pH 7.4) or 10 mM MES (pH 6.5) containing 0.1 mM EDTA, 0.1 mM PMSF, and 1 mM β-mercaptoethanol. Two hundred microliters of each protein sample (50 μg) was loaded onto the column, and eluted with a flow rate of 0.5 mL/min at room temperature. Molecular sizes of the α-synuclein and synuclein112 proteins were evaluated with reference to molecular mass standards. Standards were blue dextran (2 MDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and RNnase A (14 kDa). Same buffer with 8 M urea were used for the measurement of the elution profile of α-synuclein in the presence of urea.

Circular dichroism spectroscopy

The CD spectra were measured using a JASCO-J715 spectropolarimeter (Jasco, Japan) equipped with a temperature control system in a continuous mode. Far-UV CD measurements were carried out over the wavelength range of 190 to 250 nm, with a 0.5 nm bandwidth, 1 s response time, and 10 nm/min scan speed at 20 °C in a cuvette with a pathlength of 0.1 cm. For the CD spectra at 100 °C, the temperature was maintained using a thermostated water bath and water-jacketed sample cells were used. Spectra were smoothed using the operating software noise reduction filter and represented the average of at least four scans corrected for buffer absorbance.

α-Synuclein aggregation assay

To investigate the effects of organic solvents on α-synuclein aggregation, α-synuclein (20 μM) or α-synuclein114 (10 μM) were dissolved in 10% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (v/v), 20 mM MES pH 6.5, and incubated at 65 °C for 10 min. The turbidity was measured at 360 nm to determine the level of organic solvent-induced aggregation. To investigate the effects of different metal ions, 50 μM of α-synuclein in 20 mM MES (pH 6.5) incubated with or without metal ions (100 μM) at 65 °C for 10 min and absorbances at 360 nm were recorded. Light scattering of α-synuclein were also investigated in the presence of zinc ion every 1 min at 65 °C to determine the level of aggregation.

Chaperone-like activity of α-synuclein

Turbidity measurement as an assay for aggregation was performed according to the established protocols with minor modifications (Horwitz et al., 1998; Lee & Vierling, 1998). Protein solutions with or without α-synuclein were mixed in cuvettes at room temperature and then placed in the thermostatic cell holder, and the apparent absorbance was monitored as a function of time. Individual protein solutions of α-synuclein, aldolase (0.2 mg/mL), GST (0.2 mg/mL), or mixtures of α-synuclein with aldolase, GST at the same final concentration were prepared in phosphate-buffered saline (PBS) and heated at 65 °C. The light scattering of the solution at 360 or 400 nm was measured for total 20 min. For DTT-induced reduction, protein solution of α-synuclein, BSA (0.2 mg/mL), α-lactalbumin (0.5 mg/mL), or lysozyme (0.5 mg/mL) and mixtures of α-synuclein with BSA α-lactalbumin, or lysozyme at the same final concentration, were prepared in 10 mM phosphate pH 7,4 and incubated at 37 °C with or without 20 mM DTT (from a 100 mM stock solution). For preheating, α-synuclein was heated at 65 °C for 10 min and cooled to room temperature for 1 h. Thermally or DTT-induced aggregation of proteins was measured in a Beckman spectrophotometer fitted with thermostatic cell holder assembly with electric temperature control.

GST activity measurement

GST was prepared at a concentration of 200 μg/mL in 50 mM Na_2HPO4, pH 7.0 and incubated with or without α-synuclein (100 μg/mL) at room temperature. Mixtures were heated at 65 °C for 10 min. GST was then diluted into substrate solution (1 mM GSH, 2 mM 1-chloro-2,4-dinitrobenzene in 0.1 M phosphate pH 7.4) to a final concentration of 10 μg/mL and incubated at 37 °C for 10 min before measuring the absorbance at 350 nm. Enzyme activity was measured as an increase in absorbance, corresponding to the appearance of 1-S-glutathionyl-2,4-dinitrobenzene. The absorbance was measured on a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, California).

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