Structural and Functional Implications of C-Terminal Regions of α-Synuclein[†]

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ABSTRACT: Aggregation of α-synuclein is thought to play a major role in the pathogenesis of Parkinson's disease (PD), which is characterized by the presence of intracytoplasmic Lewy bodies (LB) in the brain. α-Synuclein and its deletion mutants are largely unfolded proteins with random coil structures as revealed by CD spectra, fluorescence spectra, gel filtration chromatography, and ultracentrifugation. On the basis of its highly unfolded and flexible conformation, we have investigated the chaperone-like activity of α-synuclein in vitro. In our experiments, α-synuclein inhibited the aggregation of model substrates and protected the catalytic activity of alcohol dehydrogenase and rhodanese during heat stress. In addition, α-synuclein inhibited the initial aggregation of reduced/denatured lysozyme on the refolding pathway. Interestingly, deletion of the C-terminal regions led to the abolishment of chaperone activity, although largely unstructured conformations are maintained. Moreover, α-synuclein could inhibit the aggregation of various *Escherichia coli* cellular proteins during heat stress, and C-terminal deletion mutants could not provide any protection to these cellular proteins. Results with synthetic C-terminal peptides and C-terminal deletion mutants suggest that the second acidic repeat, ^{125}YEMPSEEGYQDYEPEA^{140}, is important for the chaperone activity of α-synuclein, and C-terminal deletion leads to the facilitated aggregation with the elimination of chaperone activity.

Parkinson's disease (PD)[1] is a neurodegenerative disorder that predominantly affects dopaminergic nerve cells in the nigrostriatal system as well as other regions of the brain. A pathological hallmark of PD is the presence of intracytoplasmic Lewy bodies (LBs), which also accumulate in dementia with Lewy bodies (DLB) and multiple system atrophy (MSA). α-Synuclein is the principal filamentous component of Lewy bodies in PD (*1*, *2*). The importance of α-synuclein in the pathogenesis of PD is also supported by a number of animal model studies, which show the appearance of neuronal α-synuclein deposits, dopamine deficiency, and motor impairments (*3*−*5*). In addition, two mutations (A30P, A53T) in the α-synuclein gene were found in early-onset forms of PD (*6*, *7*). α-Synuclein itself spontaneously forms typical amyloid fibrils in a nucleation-dependent manner, which appears to involve prefibrillar oligomeric intermediates (*8*, *9*). These α-synuclein fibrils are stained by Thioflavin T and Congo red, and exhibit a characteristic cross-β structure, as indicated by X-ray diffraction (*10*).

The primary structure of α-synuclein (140 aa) can be described as consisting of three distinct regions: N-terminal, hydrophobic NAC, and acidic C-terminal regions. α-Synuclein binds to artificial liposomes of phospholipids with acidic headgroups. This binding seems to be mediated by the N-terminal regions of the protein, which was proposed to form an amphipathic α-helix. Recent reports include detailed analysis of the conversion from random coil to α-helical structure upon binding to small phospholipid vesicles (*11*−*13*). The integral fragment of residues 61−95, originally labeled NAC, was found to be one of the major components of the insoluble fibril core of Alzheimer's disease senile plaques (*14*). The C-terminal region, which represents a putative Ca^{2+}-binding region (*15*), is rich in acidic amino acids and shows significant differences in primary sequences among α-, β-, and γ-synucleins (*16*). Interestingly, the C-terminal-region-truncated form (residues 1−120) was more prone to form filaments than the full-length protein (*17*).

Although substantial progress has been made regarding the understanding of aggregation and fibrillation process of α-synuclein, the physiological function of α-synuclein is poorly understood (*1*, *2*, *18*, *19*). Recent NMR studies imply that the main part of the α-synuclein polypeptide is almost unfolded (*20*), which perhaps explains its ability to interact with many other proteins or ligands (*18*−*21*). In previous reports, we hypothesized that the natively unfolded conformation of α-synuclein might be used to interact with other proteins and thereby influence the intermolecular interactions required for the aggregation process (*22*, *23*). Here, our data indicate that α-synuclein could act as a molecular chaperone in vitro to protect the catalytic activity of alcohol dehydro-

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[1] Abbreviations: PD, Parkinson's disease; DLB, dementia with Lewy bodies; MSA, multiple system atrophy; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; GdmCl, guanidinium hydrochloride; ADH, alcohol dehydrogenase; PBS, phosphate-buffered saline; NAC, nonamyloid component; AFM, atomic force microscopy.

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genase and rhodanese, as well as to inhibit the aggregation of model substrate protein. In addition, α-synuclein could inhibit the aggregation of various *Escherichia coli* cellular proteins during heat stress. Interestingly, deletion mutants of the C-terminal region could eliminate the chaperone activity, although they maintain their natively unstructured conformations. It is suggested that the second acidic repeat of C-terminal regions, ^{125}YEMPSEEGYQDYEPEA^{140}, seems to be important for the chaperone activity of α-synuclein, and the C-terminal deletion leads to the facilitated aggregation of α-synuclein.

EXPERIMENTAL PROCEDURES

Materials. All peptides used in this study were synthesized at Peptron (Daejon, Korea) and purified by the reverse-phase HPLC method (purity > 95%). Protein size markers for SDS−PAGE and aldolase were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Trimethylamide-*N*-oxide was obtained from Aldrich (WI), and phosphatase inhibitor 2 was from New England Biolabs (MA). All other chemicals were purchased from Sigma (MO) unless otherwise stated.

Protein Preparation. α,*β*-Synucleins and α-synuclein 112 were overexpressed in *E. coli* and purified as previously described (*22*, *23*). Truncated forms of α-synuclein, α-synuclein 1−97, and α-synuclein 1−114 were prepared by endoprotease Asp-N digestion (*23*). The GST-SYN constructs were kindly provided by Dr. H. Lim (Catholic University, Korea). The phosphatase inhibitor-1 from rabbit skeletal muscles was purified according to the method described previously (*24*). The purities of the proteins were verified by SDS−PAGE and the protein concentrations were determined by BCA method or by direct absorbance measurements as described by Gill & von Hippel (*25*).

Circular Dichroism (CD) Spectroscopy. CD spectra were recorded with a Jasco-J715 spectropolarimeter (Jasco, Japan) equipped with a temperature control system in a continuous mode (*22*). Far-UV CD measurements were carried out over a wavelength range of 190−260 nm with a 0.5 nm bandwidth, 1 s response time, and 50 nm/min scan speed at 20 °C. All spectra comprised the average of five scans that were corrected by the subtraction of the buffer signal.

Steady-State Fluorescence Spectroscopy. All fluorescence measurements were performed with a Jasco FP-750 spectrofluorometer. The incubation buffer was composed of 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5/0.5 mM ethylenediaminetetraacetic acid (EDTA) in the presence or absence of urea.

Gel-Filtration Chromatography. To estimate the elution volume of α-synuclein and its deletion mutants, gel-filtration chromatography was performed. Each protein sample (50 μg) in 200 μL was loaded onto the Superdex 75 HR 10/30 column (Pharmacia) equilibrated with phosphate-buffered saline (PBS, pH 7.4), and the protein was eluted with a flow rate of 0.5 mL/min at room temperature. Chemically unfolded protein samples were similarly run in the same buffer containing an additional 6 M guanidinium hydrochloride (GdmCl) with 10 mM dithiothreitol (DTT). By measuring the absorbance at 280 nm/220 nm, we monitored the elution patterns of protein samples. The proteins used for calibrating the column were alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.5 kDa), and aprotinin (6.5 kDa). Blue dextran (∼2 MDa) was used to determine the void volume (Vo) of the column.

Sucrose Gradient Ultracentrifugation. Preformed sucrose gradients (5%−20% in 13 mL of PBS) were produced in Beckman UltraClear tubes and stored at 4 °C in a cold room for 2 h before use. Each protein (∼50 μg) in 200 μL was layered on top of each gradient and centrifuged in a Beckman L8-M ultracentrifuge with a SW 41Ti rotor at 40 000 rpm for 23 h at 4 °C. Fractions were collected from the bottom of each tube with an average volume of ∼0.45 mL.

Chaperone Activity of α*-Synuclein.* Molecular chaperone activity of α-synuclein was investigated according to the procedures desribed in previous reports with minor modifications (*22*, *26*). Protein solutions of α-synuclein, α-synuclein 1−97, α-synuclein 1−114, α-synuclein 112, and *β*-synuclein with aldolase were prepared in 20 mM MES, pH 6.5, and heated at 60 °C. The turbidity of the solution at 405 nm was measured using a 96-well microtiter plate after 30 min. To investigate the effect of sodium chloride on the chaperone-like activity, increasing amounts of sodium chloride up to 2 M NaCl were added to the reaction mixture.

The enzyme activity of alcohol dehydrogenase (ADH) was measured in 100 mM phosphate buffer, pH 7.0, containing 0.2 mM NAD^+ and 1 mM ethanol, and NAD^+ reduction was monitored at 340 nm (*27*). The activity was determined by taking aliquots at different times from the assay mixture. Rhodanese activity was measured by a colorimetric method based on the formation of the complex between ferric ions and thiocyanate (*28*). The assay mixture consisted of 1:1:1 (volume) of 0.25 M sodium thiosulfate, 0.25 M KCN, and 0.2 M KH_2PO_4. The reaction was stopped by adding 15% formaldehyde and developed by ferric nitrate. The capacity of different peptides to protect against heat-induced activity loss of ADH and rhodanese was measured according to the previous procedure (*29*).

Lysozyme was dissolved in buffer containing 8 M urea, 100 mM Tris-HCl and 1mM EDTA at pH 8.5. Oxidized lysozyme was refolded by direct dilution into the refolding buffer. Reduction of disulfide bonds was achieved by incubation at 37 °C with a 25-fold molar excess of DTT for 3 h (*30*). Reduced/denatured lysozyme was refolded at low concentrations by rapid dilution of a concentrated solution in formic acid (pH 2.0) into refolding buffer at pH 8.0 with 100 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA and 0.5/1 mM oxidized glutathione (GSSG)/reduced glutathione (GSH). Enzyme activities were measured at room temperature by following the decrease in absorbance at 450 nm of *Micrococcus lysodeicticus* cell well suspension in 20 mM sodium phosphate (pH 7.0). Activities were normalized to a native lysozyme solution of known concentration.

Interaction with E.coli cellular proteins. To investigate the effect of α-synuclein, glutathione-S-transferase (GST), and α-synuclein 112 on the thermal aggregation of various *E. coli* cellular proteins, cell lysates of *E. coli* BL21 (DE3) expressing each protein were heated at various temperatures (40−70 °C) for 20 min. After centrifugation at 15 000 rpm for 20 min to precipitate the denatured *E. coli* proteins, the supernatants were analyzed by SDS−PAGE. To identify the region responsible for the chaperone activity, GST-SYN(1−140)-, GST-SYN(1−60)-, GST-SYN(1−105)-, GST-

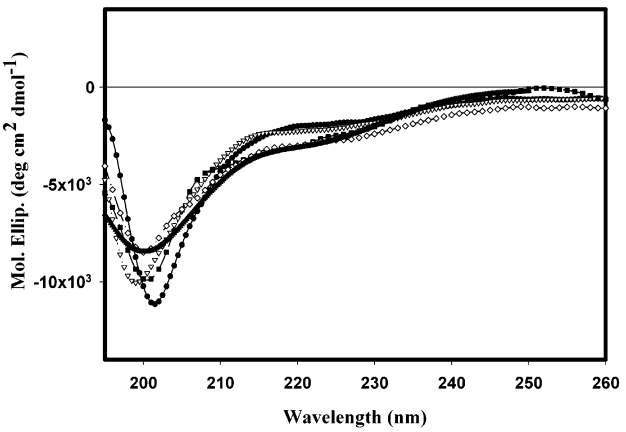

FIGURE 1: Far-UV CD spectra of α-synuclein and its deletion mutants. Far-UV CD spectra of (■) α-synuclein, (▽) α-synuclein 112, (◇) α-synuclein 97, (●) phosphatase inhibitor-1, and (▲) inhibitor-2 are shown. Two deletion mutants, α-synuclein 114 (1−114, which are not shown for clarity) and α-synuclein 97 (1−97) are prepared from enzymatic cleavage of the full-length α-synuclein. Another mutant, α-synuclein 112, is lacking 28 amino acids (102−130) within the C-terminal acidic region. Two other natively unstructured proteins of phosphatase inhibitor 1 and 2 were shown for comparison. CD spectra were measured at 20 °C, and the cell path length was 0.1 cm.

SYN(61−95)-, GST-SYN(61−140)-, and GST-SYN(96−140)-expressing cell lysates were diluted to a uniform protein concentration of 1 mg/ml. The total protein concentration was determined using the Bradford assay kit (Bio-rad, CA). The growth curves of all GST fusion protein-expressing cells were similar to that of the pGST with or without IPTG treatment (data not shown). Aggregation level of each sample was monitored by measuring the apparent light scattering at 405 nm.

Atomic Force Microscopy. For atomic force microscopy (AFM), aliquots of incubation solution were transferred to an Eppendorf tube and spun to pellet the precipitated material, which was then washed twice with water before resuspension in deionized water. Aliquots of 2−3 μL were placed on freshly cleaved mica (Ted Pella, Redding, CA) and incubated for 60 s, after which mica was gently rinsed twice with 50 μL of filtered, deionized water to remove salt and loosely bound protein and dried immediately with nitrogen gas. Images were obtained with a Nanoscope IIIa Multimode scanning probe workstation (Digital Instruments, Santa Barbara, CA) operating in tapping mode by using etched silicon NanoProbes (model FESP; Digital Instruments) (*31*).

RESULTS

Structural Conformations of α-Synuclein and its Deletion Mutants. In previous studies, we have shown that α-synuclein exists as a largely unstructured, random-coiled conformation in solution (*22*, *23*). The secondary structures of deletion mutants were determined by far-UV CD spectral analysis. All far-UV profiles showed characteristic random coil conformations with slight changes in the negative intensity around 200 nm, which resembles other already known unstructured proteins such as phosphatase inhibitor 1 and 2 (Figure 1). Deletion of C-terminal regions did not lead to significant changes in the CD spectra. Therefore, although charged amino acids often participate in the formation of largely unstructured proteins in some cases (*32*, *33*), acidic C-terminal regions in α-synuclein did not seem to have a significant role in the conformation of α-synuclein.

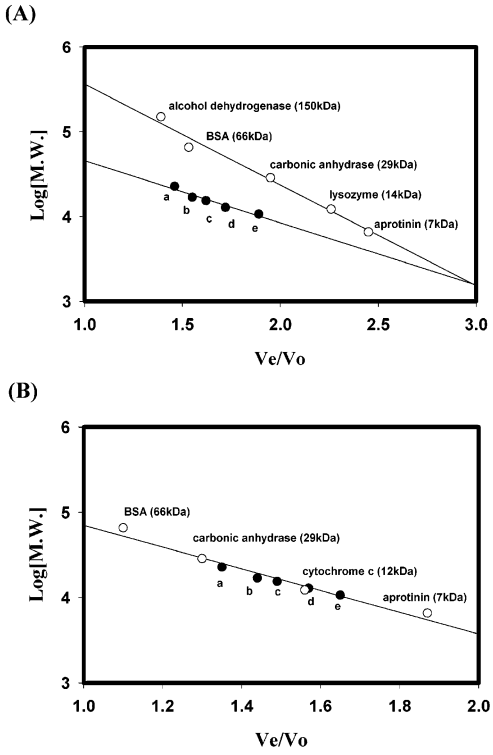

FIGURE 2: Gel filtration chromatography of α-synuclein and its deletion mutants. Gel filtration chromatography under (A) non-denaturing and (B) denaturing conditions was performed to estimate the elution volume of α-synuclein and its deletion mutants. Each protein sample was loaded onto the Superdex 75 HR 10/30 column equilibrated with PBS (pH 7.4) with a flow rate of 0.5 mL/min at room temperature. For denaturing conditions, samples were run in the phosphate buffer (50 mM phosphate buffer, pH 7.5) containing 6 M GdmCl with 10 mM DTT after 2 h incubation. By measuring the absorbance at 280 nm/220 nm, we monitored the elution volumes of protein samples: (a) phosphatase inhibitor-2 (204 aa); (b) phosphatase inhibitor-1 (171 aa); (c) α-synuclein (140 aa); (d) α-synuclein 114 (114 aa); (e) α-synuclein 97 (97 aa).

Figure 2A shows the elution behavior of α-synuclein and its deletion mutants in size exclusion chromatography (SEC). α-Synuclein and its deletion mutants eluted at larger elution volumes (Ve) than those of globular proteins in buffer solution. Therefore, the apparent molecular weight of α-synuclein under these conditions is estimated to be 59 kDa, which is equivalent to a Stokes radius of 34 Å from the standard curve. This value is as much as 1.8 times larger than the expected value (19 Å) from its molecular weight. The apparent molecular weights of α-synuclein 114 and 97 are estimated to be 42 and 31 kDa, respectively. The Ve/Vo for α-synuclein (14.4 kDa) is 1.62, while those of BSA (66 kDa) and lysozyme (14 kDa) are 1.54 and 2.26, respectively (Table 1). The Ve/Vo for α-synuclein 114 and 97 are 1.73 and 1.89. These values are consistent with highly unstructured natures, as indicated by CD spectra (Figure 1). The conformations of α-synuclein and its deletion mutants were also investigated by sucrose gradient ultracentrifugation. The frictional ratio of these unstructured proteins is in the range of 1.95−2.12 (Table 1). In denaturing conditions of 8 M urea or 6 M GdmCl, α-synuclein and its deletion mutants showed small decreases in elution volumes (Ve) with the

Table 1: Conformational States of α-Synuclein and Its Deletion Mutants[a]

	MW (kDa)	R_s[b](Å)	Ve/Vo[c]	f/f_o[d]	R_s[e](Å)	Ve/Vo
α-synuclein 97	9.6	27.3	1.89	1.95	29.0	1.65
α-synuclein 112	11.3	30.4	1.72	2.05	32.1	1.57
α-synuclein 114	11.4	30.6	1.72	2.06	32.0	1.57
α-synuclein	14.5	34.4	1.62	2.13	37.4	1.49

[a] Hydrodynamic properties of α-synuclein and its deletion mutants were determined by gel filtration chromatography experiments. [b] Stokes radii were determined from gel filtration chromatography or dynamic light scattering in phosphate buffer solution (50 mM, pH 7.5). [c] Ve is the elution volume of each protein, and Vo is the void volume of the column. [d] Frictional ratio, $f/f_o = \alpha/(3\nu M/4\pi N)^{1/3}$, where M = molecular weight, α = Stokes radius, ν = partial specific volume, and N = Avogadro's number. An averaged partial specific volume of 0.725 cm^3/g was used for calculation (*31*). [e] Stokes radii were determined in phosphate buffer with 6 M GdmCl.

increase of Stokes radii by about 2−3 Å (Table 1, Figure 2B). Under this condition, α-synuclein migrated more slowly than carbonic anhydrase (29 kDa) and α-synuclein 97 (9 kDa) migrated more slowly than lysozyme (14 kDa). The Ve/Vo for α-synuclein 114 and 97 under denaturing condition are 1.57 and 1.65, respectively, while that of lysozyme is 1.56. Addition of urea up to 8 M to α-synuclein resulted in a gradual increase of the emission intensity maxima at 305 nm (excitation at 280 nm) in spectrofluorometer without a specific midpoint (data not shown). The sequential refolding of α-synuclein with dilution of urea restores to its initial fluorescence spectra, which suggests that urea-induced changes of α-synuclein are small and reversible (data not shown).

Chaperone Activity of α*-Synuclein to Suppress Protein Aggregation.* We have previously shown that α-synuclein could prevent the heat- and chemical-induced aggregation of model substrates (*22*). Using deletion mutants, we have investigated the role of the C-terminal region on its chaperone activity. We have incubated aldolase with α-synuclein deletion mutants, and aggregation levels were measured. Interestingly, C-terminal deletion mutants did not show any significant suppression of the aggregation at concentrations at which full-length α-synuclein proved to be very effective (Figure 3A). When α-synuclein 97 and aldolase were coincubated, over 90% of the aggregation of aldolase was not inhibited compared to wild-type protein. Similarly, α-synuclein 112 or 114 did not effectively inhibit the aggregation of aldolase. When the same experiment was performed in the presence of *β*-synuclein with similar acidic C-terminal regions to α form, aggregation was held back to a level similar to that with α-synuclein (Figure 3A, Table 2). Therefore, it seems that the C-terminal regions are necessary for the chaperone activity, while N-terminal hydrophobic regions are not important for this function. Considering the fact that the C-terminal regions are enriched with acidic amino acids, we have investigated the effects of salt addition that affect electrostatic interactions of α-synuclein/substrate complex. Addition of NaCl to the reaction mixtures led to a gradual increase of the level of protein aggregation, which implied that salt addition could weaken or inhibit the formation of α-synuclein/substrate complex. As shown in Figure 3B, in the presence of 400 mM NaCl, aldolase aggregation was increased up to 20%. Similar behavior was also observed for the addition of KCl (data not shown). Therefore, we speculate that many acidic residues in C-terminal regions might play an important role in the chaperone activity of α-synuclein.

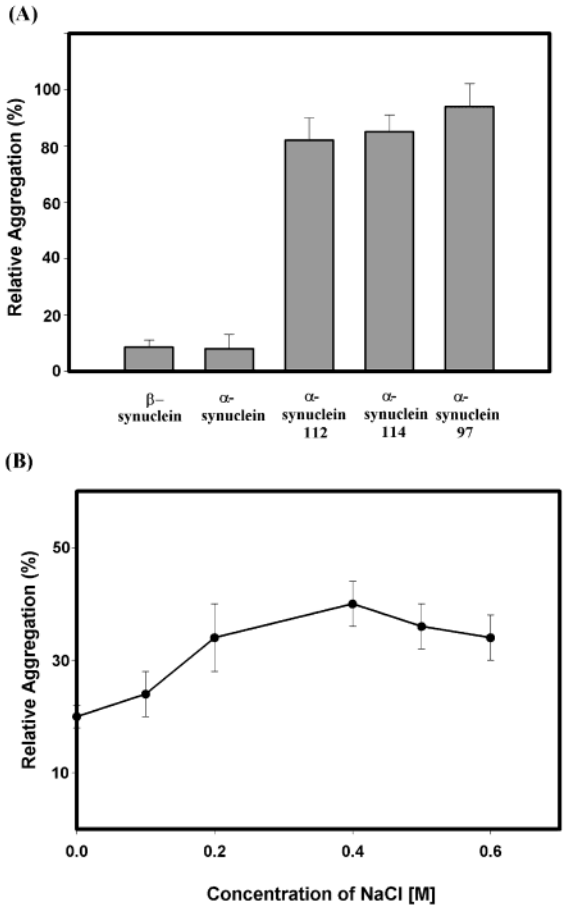

FIGURE 3: Effects of α,*β*-synuclein and deletion mutants on the thermal aggregation of aldolase. Panel A presents the effects of α,*β*-synuclein and its deletion mutants on the aggregation of aldolase during its thermal denaturation at 60 °C. Two micromolar of aldolase and 3.5 μM of α,*β*-synuclein and its deletion mutants were coincubated for 10 min, and the absorbance at 405 nm was measured. In panel B, the effect of NaCl on the aggregation of aldolase was investigated. Two micromolar of aldolase and 3.5 μM of α-synuclein with increasing concentrations of NaCl were used for the aggregation experiments.

Table 2: Effects of Peptides of α-Synuclein on Chaperone Activity[a]

peptide name	sequence[b]	relative scattering (%) lysozyme	ADH
NAC	^{71}VTGVTAVAQKTA^{82}	94	97
TEM 1	^{109}Q**E**GIL**ED**MPV**D**P**D**N**E**A^{124}	72	65
TEM 2	^{125}**YE**MPS**EE**G**Y**Q**D**Y**E**P**E**A^{140}	53	47
β-SYN	^{119}**YE**DPDQ**E**E**Y**Q**E**Y**E**P**E**A^{134}		
14-3-3	^{209}L**D**TLG**EE**S**Y**K**D**STLI^{223}		

[a] Effects of peptides on anti-aggregation assay of ADH and lysozyme. Heat-induced aggregation assay of ADH and lysozyme were performed as described previously (*22*). [b] Conserved amino acids of special interests are shown in bold type.

Here, we have also investigated whether α-synuclein might protect the enzymatic activity of substrate proteins using alcohol dehydrogenase (ADH) and rhodanese during heat stress. As shown in Figure 4A, ADH slowly loses its activity during heat stress, and the loss was about 70% upon incubation for 50 min at 60 °C (trace 1). However, when the same experiment was performed in the presence of

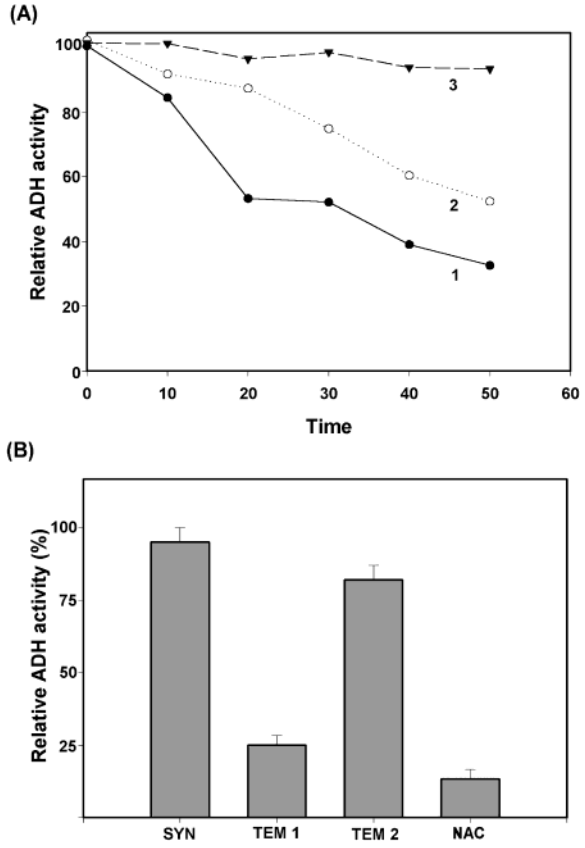

FIGURE 4: Effects of α-synuclein and its peptides on the loss of enzymatic activity of alcohol dehydrogenase. Panel A presents the effect of α-synuclein on the enzyme activity of ADH during thermal denaturation at 60 °C: (●, trace 1) ADH alone; (○, trace 2) ADH with 3 μM α-synuclein; (▼, trace 3) ADH with 6 μM α-synuclein. Panel B presents the effects of α-synuclein (SYN) and peptides (TEM 1, TEM 2, NAC) on the activity of ADH during thermal denaturation at 60 °C. Thermal protection of ADH activity by α-synuclein was set to 100%.

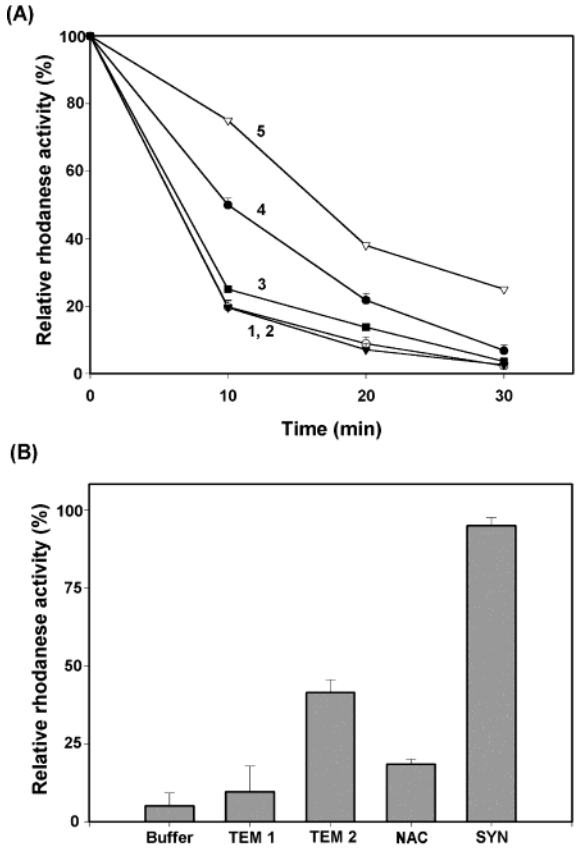

FIGURE 5: Effects of α-synuclein and its peptides on the loss of enzymatic activity of rhodanese. Panel A presents the effect of α-synuclein on the enzyme activity of rhodanese during thermal denaturation at 60 °C: (▽, trace 5) rhodanese with 10 μM α-synuclein; (●, trace 4) rhodanese with 3 μM α-synuclein; (■, trace 3) rhodanese with buffer only; (○, trace 2) rhodanese with 3 μM BSA; (▼, trace 1) rhodanese with 3 μM α-lactalbumin. Panel B presents the effects of α-synuclein (SYN) and peptides (TEM 1, TEM 2, NAC) on the activity of rhodanese during thermal denaturation at 60 °C. Thermal protection of rhodanese activity by α-synuclein was set to 100%.

α-synuclein, the enzyme activity was protected (traces 2, 3). Specifically, when ADH and α-synuclein (6 μM) were co-incubated, the enzyme retained nearly 90% of its activity in this condition (trace 3), which behavior is not observed for BSA (1 mg/ml) or bovine α-lactalbumin (1 mg/ml)(data not shown). In the control experiments, it was found that the addition of α-synuclein to ADH did not influence the specific activity of the enzyme. The peptide TEM 2 (corresponding to the amino acid residues of 125−140) was able to prevent loss of enzyme activity up to 80% of wild-type α-synuclein (Figure 4B). However, a peptide TEM 1 (residues 109−124), which was presumed to be one of the regions responsible for the Ca^{2+} binding (*15*), was less effective than TEM 2 in suppressing the loss of enzyme activity. As expected, hydrophobic peptide NAC (residues 71−82) was inactive as a chaperone peptide. Similar observations were made with rhodanese (Figure 5A). In this case, heating 2.5 μM rhodanese alone at 60 °C caused a severe loss of enzyme activity in less than 30 min. However, in the presence of 3 and 10 μM α-synuclein, loss of enzyme activity was effectively prevented (traces 4, 5). This is not observed for BSA (1 mg/ml) or bovine α-lactalbumin (1 mg/ml)(traces 1, 2). Again, the peptide TEM 2 was able to prevent loss of enzyme activity of rhodanese more effectively than TEM 1 (Figure 5B). These results suggest that α-synuclein (especially through TEM 2 region) binds ADH and rhodanese during thermal stress and protects them from loss of enzymatic activity. These results are similar to those obtained for many other chaperones (*34*−*36*).

Role of α*-Synuclein in Lysozyme Refolding.* It has been previously shown that α-synuclein could prevent the chemical-induced aggregation of lysozyme in the unfolding pathway (*22*). Here, we have investigated whether α-synuclein could bind to refolding intermediates by measuring the biological activity of refolded lysozyme. The BSA and α-lactalbumin are well suited as background species to monitor any nonspecific interactions, because BSA and α-lactalbumin have a similar Stokes radius and molecular weight to α-synuclein (Figure 2). When denatured lysozyme is refolded from the buffer containing 8 M urea, the refolding yields are hardly affected by the presence or absence of α-synuclein (Figure 6A). Therefore, α-synuclein did not show any significant effect on the renaturation yield of denatured lysozyme, and this is also the same for the case of BSA, bovine α-lactalbumin, and α-crystallin**,** because the refolding of oxidized lysozyme is rapid and essentially complete after a few seconds (*37*−*39*).

Interestingly, although refolding of denatured-reduced lysozyme (i.e., all disulfide bonds were broken) without GSH/GSSG resulted in aggregation, refolding it only with

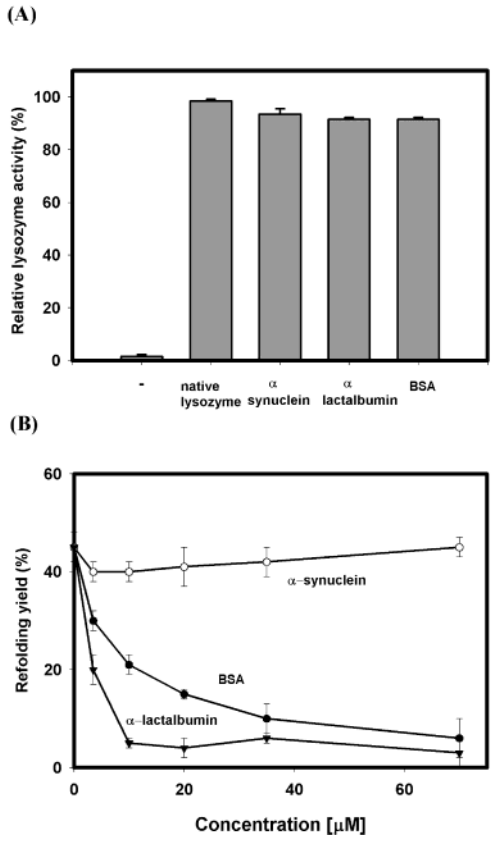

FIGURE 6: Effects of α-synuclein, α-lactalbumin and BSA on lysozyme refolding. In panel A, hen egg white lysozyme (7 μM), denatured in the buffer containing 8 M urea, 100 mM Tris-HCl, 1 mM EDTA, pH 8.5, was refolded by direct dilution in the refolding buffer containing 100 mM sodium phosphate, pH 8.0, 100 mM NaCl, 1 mM EDTA, containing 0.5/1 mM GSSG/GSH. Each protein (20 μM of α-synuclein, α-lactalbumin, or BSA) was included in the refolding buffer. The symbols (−) and (+) represent negative and positive control. In panel B, reduced/denatured lysozyme (7 μM) was refolded in the presence of varying concentrations of (○) α-synuclein, (●) BSA, and (▼) α-lactalbumin. Enzyme activities were measured at room temperature by following the decrease in absorbance at 450 nm of a *Micrococcus lysodeicticus* cell well suspension in 20 mM sodium phosphate (pH 7.0).

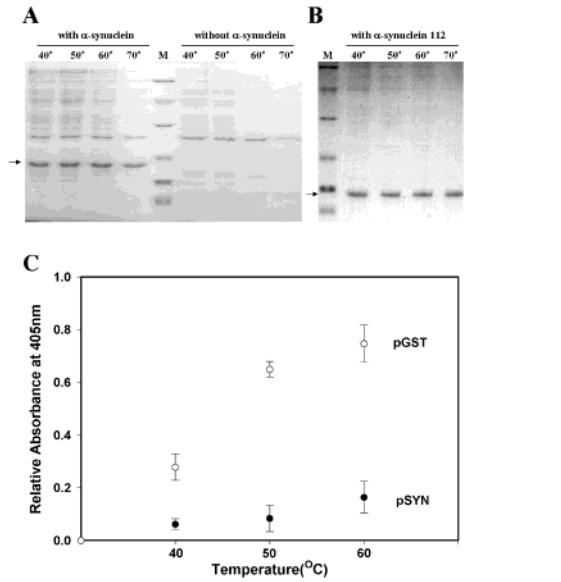

FIGURE 7: SDS−PAGE analysis of bacterial soluble proteins during heat shock at 40, 50, 60, and 70 °C, showing the effects of (A) α-synuclein and (B) α-synuclein 112 on the thermal aggregation of *E. coli* soluble cytoplasmic proteins in vitro. *E. coli* cell extracts expressing α-synuclein and α-synuclein 112 were heated at various temperatures (40−70 °C) for 20 min. After centrifugation, the soluble fractions were analyzed by SDS−PAGE. The M lane represents 66, 45, 31, 21, 11, and 7 kDa size of proteins. In panel C, *E. coli* cell extracts expressing α-synuclein and GST were heated at 50 °C. Aggregation of each sample was monitored by measuring apparent light scattering at 405 nm.

α-synuclein results in the much less aggregation (data not shown). In addition, as shown in Figure 6B, there is a marked difference between the action of the control proteins and α-synuclein when the oxidative refolding was undertaken. Refolding lysozyme with increasing concentrations of BSA or α-lactalbumin led to a large increase in the measured turbidity of the solution with a concomitant decrease in the refolding yield, as reported earlier (*37*). However, α-synuclein inhibits the initial aggregation through binding to refolding intermediates of lysozyme, although we could not detect any significant increase of lysozyme refolding yield as observed in α-crystallin or clusterin (*37*, *40*). This observation suggests that α-synuclein could act as a passive chaperone in the refolding process. It is not directly able to increase the refolding yields, but it can only reduce the initial aggregation of lysozyme, which might be used to save time for correct refolding by other chaperone networks in a crowded cellular environment.

Interaction with E. coli Cellular Proteins. To investigate whether α-synuclein can protect *E. coli* cellular proteins from precipitation during heat stress, we analyzed the stability of *E. coli* cellular proteins from control cells and pSYN (α-synuclein-expressing) cells following heat treatment at 40, 50, 60, and 70 °C for 20 min (Figure 7A). Heat treatment of lysates of *E. coli* control cells leads to the aggregation of cellular soluble proteins at 40 °C, while many cellular proteins remain soluble up to 60 °C in pSYN cell lysates. Interestingly, analysis of all of the extracted cellular proteins from pSYN112 cells showed that the presence of α-synuclein 112 did not significantly affect the thermostability of other cellular proteins, although α-synuclein 112 is very stable during heat stress (Figure 7B). The pSYN112 cells produced a variant of α-synuclein without large portions of C-terminal acidic regions (amino acid residues 103 through 130). Therefore, we speculated that α-synuclein 112 was unable to confer thermoprotection because of its disability to interact effectively with other *E. coli* cellular proteins resulting from the absence of C-terminal regions. This is consistent with the reduced chaperone activity of α-synuclein 112 (Figure 3A). In accordance with this finding, protein turbidity assays demonstrated that α-synuclein had shown nonspecific interactions with *E. coli* cellular proteins to prevent aggregation in vitro. Incubation at 50 °C only led to a slight rise in the level of light scattering of pSYN cell lysates, indicating that α-synuclein was able to suppress thermal aggregation of *E. coli* cellular soluble proteins (Figure 7C). However, the relative level of light scattering of pGST cell lysates increased significantly, which suggested that the observed thermoprotection was not due to the stress response by overproduction of foreign proteins. This analysis was also performed on all the proteins extracted from *E. coli* cells expressing GST-fusion proteins of α-synuclein deletion mutants. Incubation at 50 °C had only little effects on the level of light scattering in GST-SYN(1−140)-, GST-SYN(61−140)-, and GST-SYN(96−140)-expressing cell lysates, indicating that acidic

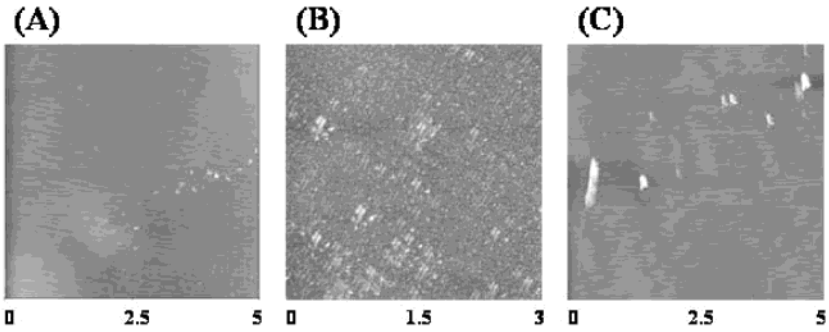

FIGURE 8: AFM images of aggregates of α-synuclein and its mutants. For AFM images, samples were prepared by placing a droplet of a solution of the protein on freshly cleaved graphite, and they were kept in a desiccator with dry nitrogen for at least 30 min: (A) α-synuclein; (B) spherical protofibrils of α-synuclein with enlarged view; (C) α-synuclein 97. All samples were incubated at 37 °C for 24 h with agitation. The areas and heights of the images are 25 μm^2 and 20 nm, respectively, for panels A and C. The areas and heights of the image in panel B are 9 μm^2 and 20 nm.

C-terminal regions were sufficient for suppressing the thermal aggregation of *E. coli* cellular proteins (data not shown).

Atomic Force Microscopy. The atomic force microscope (AFM) images allow direct visualization of the aggregates formed by the α-synuclein under various conditions. Figure 8 shows AFM images of the aggregates formed by α-synuclein adsorbed on a mica surface after 24 h shaking at 37 °C. When incubated at 37 °C, the small circular nuclei that have been reported to precede amyloid fibril in α-synuclein (*41*) are observed (Figure 8A). These spherical particles are similar to those previously reported for Aβ (*42*). Interestingly, C-terminal deletion mutant exhibited larger aggregates compared to the full-length protein under this condition (Figure 8C). Therefore, it can be suggested that the acidic C-terminal region might be involved in the aggregation process of α-synuclein.

DISCUSSION

α-Synuclein has been shown to be a major component of Lewy bodies (LB) in Parkinson's disease (PD), which implies that the aggregation of α-synuclein plays a major role in PD (*1*, *2*, *18*, *19*, *43*). Although significant advances have been made regarding the aggregation and fibrillation process of α-synuclein, the physiological function of α-synuclein is poorly understood. The conformation of α-synuclein is markedly different from most other globular proteins in that its native state is largely unstructured in solution (*21*, *22*). Therefore, α-synuclein has a large chemical potential to associate with various substrates with posttranslational modification (*18*, *19*). Recently, it has been shown that α-synuclein could function as a molecular chaperone in vitro to prevent thermal or chemical aggregation of model substrates (*22*, *26*). In this study, we have demonstrated that α-synuclein not only prevented the aggregation of model substrates, it also protected from loss of the enzyme activity of alcohol dehydrogenase and rhodanese during heat stress. Moreover, it could reduce the initial aggregation and therefore help the refolding process of lysozyme, which is not observed with BSA or α-lactalbumin. Interestingly, deletion of C-terminal regions led to a significantly reduced level of molecular chaperone activity. From biophysical analysis, all C-terminal deletion mutants of α-synuclein were capable of forming natively unstructured conformations, revealing that deletion of the C-terminal regions did not lead to large structural reorganization in secondary structure contents, molecular shape, and size. Gel filtration and sedimentation experiments have confirmed that α-synuclein and its deletion mutants exist as monomers under the conditions in the chaperone assay. The C-terminal truncation did not apparently affect the folding of the N-terminal domains of α-synuclein. Thus, chaperone activity can be attributed to the roles of the C-terminal regions of α-synuclein. In addition, protein turbidity assays demonstrated that full-length α-synuclein, but not C-terminal deletion mutants, showed nonspecific interactions with *E. coli* cellular proteins in vitro (data not shown). The C-terminal deletion mutants did not afford further protection to other heat-labile proteins during heat stress, and deletion of C-terminal regions actually facilitated the aggregation process of α-synuclein. Therefore, it is clear that the unstructured conformation of α-synuclein is necessary but not sufficient for its chaperone activity. In support of these results, many studies have shown that the C-terminal region of the α-synuclein is important for the ability to interact with other binding partners (*44*, *45*). In addition, the ability of α-synuclein to prevent aggregation and protection of enzymatic activity loss occur in the absence of ATP. In these points, α-synuclein is different from other chaperone proteins such as GroEL, Hsp18, α-crystallin, and ClpB (*46*−*49*).

The C-terminal region of α-synuclein is organized as 16 amino acids repeats, which could be important for Ca^{2+} binding (*15*) and in 14-3-3 proteins (*50*). Although it is interesting to note the concordance in these repeats, the residues located in the second repeat (^{125}YEMPSEEGYQDYEPEA^{140}) seem to be important for the chaperone activity. This region has three tyrosine residues with the many acidic amino acids that are highly conserved in α- and β-synucleins (*15*, *19*). Interestingly, it has been shown that the deletion of this second repeat reduced Ca^{2+} binding to a level of lacking all C-terminal regions (*15*). Modifications of this second repeat region are implicated in the aggregation process and protein−protein interactions of α-synuclein. For example, phosporylation of Ser^{129} could promote the formation of α-synuclein filaments or oligomers (*51*). In addition, nitration of Tyr^{125}, Tyr^{133}, and Tyr^{136} is also observed in Lewy bodies in PD and could promote α-synuclein aggregation (*52*, *53*). It is interesting to note that aromatic Phe was found to be essential for the chaperone-like activity of α-crystallin, and mutation of Phe by Gly could lose its activity without changes in molecular size or fluorescence (*54*). Therefore, we speculate

that hydrophobic residues with acidic amino acids in the second repeat region might directly be involved protein−protein interactions during chaperone property. It is also possible at present that other conserved residues in the C-terminal regions of α-synuclein could play an important role in chaperone activity. Therefore, it would be interesting to study the role of each residue in conjunction with post-translational modifications on the function of α-synuclein.

The propensity of α-synuclein to undergo large and potentially deleterious conformational changes may have important implications for its function and aggregation (*18*, *32*, *55*). In its natively unfolded state, α-synuclein is soluble enough to prevent the aggregation of other nonnative polypeptides such as other molecular chaperones. However, aggregation of α-synuclein itself might occur, for example, when α-synuclein is exposed to an abnormal concentration of ligands such as metal ions (*22*, *23*) or experiences the modulation of C-terminal regions by posttranslational modifications or deletion (*17*, *51*, *52*). These changes might be closely associated with the regulation of protein−protein interactions and chaperone activity (*56*, *22*). Taking into account the observation that A53P or A30T mutations have little effect on the conformations (data not shown), these substitutions seem not to affect the intrinsic stability of α-synuclein but only to alter the stability of the aggregation-prone state or the process leading to aggregation. Recently, it has been reported that *β*-synuclein inhibited the aggregation of α-synuclein in a transgenic mouse model or in in vitro condition (*57*, *58*). Although α-synuclein exhibits chaperone activity in addition to the aggregation property, *β*-synuclein has only a chaperone activity without hydrophobic NAC regions. Therefore, in vivo or in vitro conditions in which α-synuclein is expected to aggregate, *β*-synuclein could inhibit the aggregation of α-synuclein effectively as a chaperone. This is clearly relevant to our experiments described here and highlights the importance of the chaperone activity of the α- and *β*-synuclein family.

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