Stress-Induced Aggregation Profiles of GST−α-Synuclein Fusion Proteins: Role of the C-Terminal Acidic Tail of α-Synuclein in Protein Thermosolubility and Stability†

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ABSTRACT: α-Synuclein is a well-known heat-resistant protein that does not aggregate upon heat treatment, whereas glutathione *S*-transferase (GST) is a heat-labile protein that easily precipitates as a result of thermal stress. This paper reports the role of the C-terminal acidic tail of α-synuclein in protein thermosolubility and stability. The region of α-synuclein that is responsible for the heat resistance was initially investigated using a series of deletion mutants, and the C-terminal acidic tail (residues 96−140) was found to be crucial for the thermosolubility of α-synuclein. The thermal behavior of the GST−α-synuclein fusion protein was next investigated, and the fusion protein was seen to be extremely heat-resistant. Using a series of GST−synuclein deletion mutants, the C-terminal acidic tail of α-synuclein was shown to play a critical role in conferring the heat resistance of the fusion proteins. Furthermore, the acidic tail appeared to protect the fusion protein from pH- and metal-induced protein aggregation, suggesting that the acidic tail can increase the virtual stability of the protein by protecting it from the aggregation induced by environmental stresses. Interestingly, the acidic tail also appeared to protect the GST enzyme from the thermal inactivation to a considerable extent. However, CD analysis of the heat-induced secondary structural changes of the GST−α-synuclein fusion protein revealed that the fusion protein is irreversibly denatured by heat treatment with a slightly lowered melting temperature (T_m). Thus, the results demonstrate that introducing an acidic tail into GST promotes the thermosolubility and virtual stability of the fusion protein, although it might be unfavorable for its intrinsic stability.

Most proteins are unfolded and in turn precipitate as the temperature is increased, and the process is usually irreversible (*1*). Although most proteins are so heat-labile, some proteins are known to be stable even at the boiling temperature of water. One group of heat-stable proteins is represented by proteins from hyperthermophilic organisms (reviewed in refs *2*−*4*). These proteins have an extremely high melting temperature (T_m),[1] relative to their mesophilic counterparts (near or above the boiling point of water). However, when the temperature is increased above the T_m, most hyperthermophillic proteins also denature, leading to insoluble aggregation (*5*−*7*). Another group of heat stable proteins, which have been recently recognized, consists of the "intrinsically unstructured proteins" (also called "natively unfolded proteins"; reviewed in refs *8*−*10*). The heat stability of intrinsically unstructured proteins originates from the fact that heat treatment causes no drastic change in the conformation of the intrinsically unstructured proteins. Thermodynamically, the intrinsically unstructured proteins are not heat-stable since the conformation of the protein is almost unfolded at room temperature and must be somewhat changed at high temperatures (*11*, *12*). Thus, the term "heat-resistant proteins" (HRPs) is more appropriate for describing the thermal behavior of the intrinsically unstructured proteins. HRPs can be defined as proteins that are not aggregated by heat treatment, such as hyperthermophilic proteins and unstructured proteins.

Previously, the thermal behavior of proteins was systematically investigated by purifying and characterizing some HRPs from Jurkat T cells and human serum that are not aggregated by heat treatment (*11*). Many proteins in both Jurkat cell lysates and human serum appeared to be heat-resistant, and a systematic investigation of the effect of heat on the purified HRPs revealed four major types of thermal behavior of HRPs, indicating that protein heat resistance can be achieved in several different ways. Group I HRPs are represented by unstructured proteins such as α-synuclein and α_s-casein. These proteins have a semi-unfolded conformation regardless of temperature, although they are known to undergo some structural change at high temperatures as shown by CD (*11*, *12*). Group II HRPs represented by human serum fetuin and albumin are characterized by an irreversible

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[1] Abbreviations: HRPs, heat-resistant proteins; GST, glutathione *S*-transferase; CD, circular dichroism; T_m, melting temperature.

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conformational change upon heat treatment, while group III HRPs represented by transthyretin and bovine serum fetuin are characterized by a reversible conformational change. Group IV HRPs represented by conventional heat-stable proteins such as hyperthermophilic proteins are characterized by the absence of heat-induced conformational changes.

α-Synuclein, which is an acidic presynaptic protein of 140 amino acids (*13*, *14*), belongs to the intrinsically unstructured protein family (*15*−*17*). α-Synuclein consists of three distinct regions (reviewed in refs *18*−*21*): the N-terminal amphipathic region (residues 1−60), the hydrophobic NAC region (residues 61−95), and the C-terminal acidic tail (residues 96−140). The N-terminal region is highly conserved between species, while the C-terminal region is highly variable in size as well as in sequence. α-Synuclein is intrinsically unstructured in its native state (*16*, *17*), which may explain its ability to interact with many other proteins or ligands. Interestingly, α-synuclein acquires an increased level of secondary structure, when it associates with small acidic phospholipid vesicles, detergents, organic solvents, and some metal ions (*15*−*17*, *22*−*25*). As mentioned above, α-synuclein is extremely heat-resistant, which is possibly due to the abnormal primary and tertiary structural features.

In this study, stress-induced aggregation profiles of GST−α-synuclein fusion proteins made of GST and a series of deletion fragments of α-synuclein have been investigated, focusing on the role of the C-terminal acidic tail of α-synuclein in protein thermosolubility and stability.

MATERIALS AND METHODS

Materials. Glutathione (GSH), dithiothreitol (DTT), 1-chloro-2,4-dinitrobenzene (CDNB), and isopropyl *β*-D-thiogalactopyranoside (IPTG) were purchased from Sigma (St. Louis, MO). Glutathione−Sepharose 4B beads were obtained from Peptron (Taejeon, Korea). Bovine plasma thrombin was supplied by Sigma. Leupeptin, pepstatin, and phenylmethanesulfonyl fluoride (PMSF) were purchased from Boeringer Mannheim (Mannheim, Germany). The pGEX vector was from Pharmacia Biotech (Buckingamshire, U.K.).

Purification of α-Synuclein and GST Protein. 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 techniques, as previously described (*16*, *26*). The GST protein encoded by the pGEX expression vector was purified by affinity chromatography using glutathione−Sepharose 4B beads. The GST protein was further purified on a FPLC gel filtration column.

GST−Synuclein Fusion Constructs. A series of GST−α-synuclein fusion constructs shown in Figure 1B were generated by PCR amplification of the α-synuclein gene with the specific primer sets described below. The protein coding regions of the full-length α-synuclein (residues 1−140) and the amino-terminal amphipathic part (residues 1−60) were amplified by PCR with the 5′-oligonucleotide primer GCGCTCGAGCC**AGATCT**GCCATGGATGTATTCATGA containing the underlined *Bgl*II restriction site and 3′-oligonucleotide primers GCGCAAGCTT**GTCGAC**TTAGGCTTCAGGT TCGTAGT and GCGCAAGCTT**GTCGAC**CTATTTGGTCTTCTCAGCCAC containing the underlined *Sal*I restriction sites, respectively. The protein coding regions

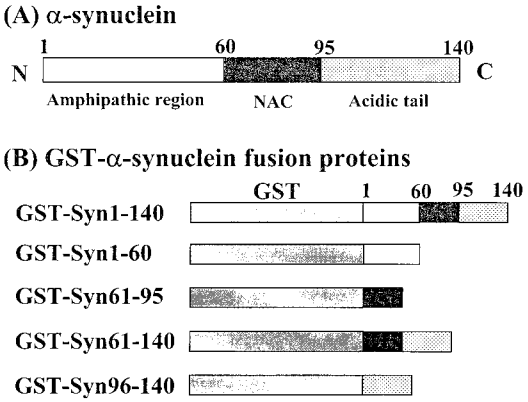

FIGURE 1: α-Synuclein and the GST−α-synuclein fusion constructs. (A) A schematic diagram of α-synuclein. α-Synuclein consists of three distinct regions: the N-terminal amphipathic region (residues 1−60), the hydrophobic NAC region (residues 61−95), and the C-terminal acidic tail (residues 96−140). (B) GST−synuclein fusion constructs. Five GST−synuclein fusion constructs encoding the full-length α-synuclein (GST−Syn1−140), the amphipathic region (GST−Syn1−60), the NAC region (GST−Syn61−95), the NAC and acidic tail regions (GST−Syn61−140), and the acidic tail region (GST−Syn96−140) were used in this study.

of the NAC (residues 61−95) and the NAC plus acidic tail (residues 61−140) were amplified by PCR with the 5′-oligonucleotide primer GCGC**AGATCT**CATATGGAGCAAGTGACA containing the underlined *Bgl*II restriction site and 3′-oligonucleotide primers GCGCAAGCTT**GTCGAC**CTAGACTTAGCCAGTGGC and GCGCAAGCTT**GTCGAC**TTAGGCTTCAGGTTCGTAGT containing the underlined *Sal*I restriction site, respectively. The protein coding region of the C-terminal acidic tail (residues 96−140) was amplified by PCR with the 5′-oligonucleotide primer GCGC**GGTACC**GAGATCTGGATGAAAAAGGACCAGTTGGGC containing the underlined *Kpn*I restriction site and 3′-oligonucleotide primer GCGCAAGCTT**GTCGAC**TTAGGCTTCAGGTTCGTAGT containing the underlined *Sal*I restriction site. The amplified DNAs were gel purified, digested with appropriate enzymes, then ligated into the pGEX 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 GST−Synuclein Fusion Proteins. The GST−synuclein fusion constructs were transformed into *E. coli* strain BL21(DE3) plysS, 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, and then cultured for a further 4 h after being induced with 0.5 mM IPTG. The cells were harvested by centrifugation at 10 000 rpm for 10 min, resuspended in phosphate-buffered saline (PBS, pH 7.4), and then disrupted by ultrasonication. After removal of the cell debris, the supernatants were loaded onto a glutathione−Sepharose 4B column equilibrated with PBS. After being washed with PBS, the protein was eluted with 10 mM GSH. The protein was concentrated and buffer changed with a Centricon apparatus (Amicon, Beverly, MA).

Heat-Induced Protein Aggregation Assay. The heat-induced aggregation of α-synuclein, GST, and GST−α-synuclein fusion proteins was qualitatively assayed by SDS−PAGE after heat treating the protein samples. Each protein in PBS (0.6 mg/mL) was heated in a boiling water bath for

10 min and cooled in the air. The protein samples were centrifuged at 15 000 rpm for 10 min, and the supernatants were analyzed on a 12% SDS−polyacrylamide gel. The protein bands were stained with Coomassie Brilliant Blue R250. The level of heat-induced aggregation of GST−α-synuclein fusion proteins was also quantitatively measured by monitoring the apparent absorbance (scattering) at 360 nm as a function of time at 65 °C (*27*, *28*). Each protein was diluted to a final concentration of 0.2 mg/mL in PBS or Tris buffer [20 mM Tris-HCl (pH 7.4)]. The protein sample in the spectrophotometric cuvette was placed in a thermostatic cell holder, and the apparent absorbance was monitored in a Beckman spectrophotometer.

pH- and Metal-Induced Protein Aggregation Assay. The pH-induced aggregation of GST and the GST−Syn96−140 fusion protein was similarly measured by monitoring the apparent absorbance (scattering) at 360 nm as a function of pH. Each protein was diluted to a final concentration of 0.2 mg/mL in buffers with different pH values. The buffers that were used were 0.1 M acetate (pH 4.0 and 5.0), 0.1 M citrate (pH 6.0), and 0.1 M Tris-HCl (pH 7.4). The protein solutions were incubated for 1 h at room temperature, and the apparent absorbance was monitored in a Beckman spectrophotometer. The metal-induced aggregation of GST and the GST−Syn96−140 fusion protein was similarly assessed. Each protein was diluted to a final concentration of 0.2 mg/mL in 20 mM Tris-HCl buffers (pH 7.4) containing 0−1.0 mM Zn^{2+} or Cu^{2+}. The protein solutions were incubated for 30 min at room temperature, and the apparent absorbance at 360 nm was measured.

GST Activity Assay. The enzymatic activity of the GST and GST−synuclein fusion proteins was assayed using a chromogenic substrate, 1-chloro-2,4-dinitrobenzene (CDNB), as previously described (*29*). The purified GST and GST−synuclein fusion proteins were diluted into the substrate solution [1 mM GSH and 2 mM CDNB in 0.1 M phosphate buffer (pH 7.4)] to a final concentration of 20 μg/mL and incubated at 37 °C for 10 min. The enzyme activity was measured as an increase in absorbance of 350 nm, corresponding to the appearance of 1-*S*-glutathionyl-2,4-dinitrobenzene. The absorbance was measured on a Spectramax 250 microplate reader (Molecular Devices).

CD Measurements. The CD spectra were recorded on a Jasco-J715 spectropolarimeter (Jasco) equipped with a temperature control system in a continuous mode. The far-UV CD measurements were carried out over the wavelength range of 190−250 nm with a bandwidth of 0.5 nm, a response time of 1 s, and a scan speed of 10 nm/min at 25 and 100 °C. The spectra shown are an average of five scans that were corrected by subtraction of the buffer signal. The CD data were expressed in terms of the mean residue ellipticity, [θ], in degrees per square centimeter per decimole. The protein samples for CD measurements were prepared in 10 mM sodium phosphate buffer (pH 7.5) unless otherwise specified, and all spectra were measured in a cuvette with a path length of 0.1 cm. The protein concentration was 0.1 mg/mL.

Thermal denaturation experiments were performed using a heating rate of 1 °C/min and a response time of 1 s. The thermal scan data were collected from 25 to 100 °C in 0.1 cm path length cuvettes with protein concentrations of 0.1 mg/mL GST and 0.3 mg/mL GST−Syn96−140. The CD spectra were measured every 0.5 °C at a wavelength of 222 nm, unless otherwise specified. The reversibility of the thermal transition was examined by recording a new scan by decreasing the temperature and by another scan after cooling the thermally unfolded protein sample.

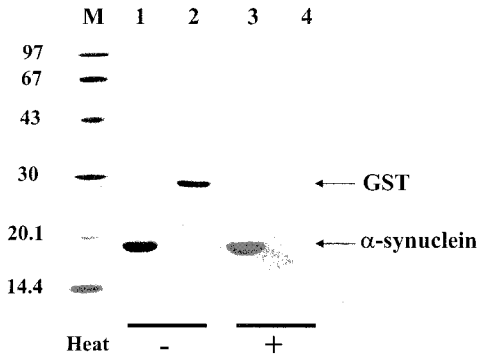

FIGURE 2: Thermal behavior of α-synuclein and the GST protein. Each protein in PBS (0.6 mg/mL) was heated in a boiling water bath for 10 min and centrifuged at 15 000 rpm for 10 min. The supernatants were analyzed on a 12% SDS−polyacrylamide gel, and the protein bands were stained with Coomassie Brilliant Blue R-250: lanes 1 and 3, α-synuclein with and without heat treatment, respectively; and lanes 2 and 4, GST with and without heat treatment, respectively.

RESULTS

Preparation of GST−Synuclein Fusion Proteins. α-Synuclein consists of three distinct regions: the N-terminal amphipathic region (residues 1−60), the hydrophobic NAC region (residues 61−95), and the C-terminal acidic region (residues 96−140; Figure 1A). Five GST−synuclein fusion constructs encoding the entire region of α-synuclein (GST−Syn1−140), the amphipathic region (GST−Syn1−60), the NAC region (GST−Syn61−95), the NAC and acidic tail regions (GST−Syn61−140), and the acidic tail region (GST−Syn96−140) were synthesized (Figure 1B). Using these constructs, the GST−synuclein fusion proteins were overexpressed in *E. coli* and purified by affinity chromatography using a glutathione−Sepharose 4B column. The GST−synuclein fusion proteins were further purified on a gel filtration column. GST protein encoded by the pGEX vector was similarly prepared. The recombinant α-synuclein was prepared as previously described (*16*, *26*). The protein samples used in this study were highly purified as determined by SDS−PAGE (shown in Figures 2−4).

Thermal Behavior of α-Synuclein and GST Protein. α-Synuclein is an intrinsically unstructured protein (reviewed in refs *8*−*10*) which almost lacks a regular secondary structure and contains a very high proportion of random coil (*16*, *17*). Previous studies have shown that intrinsically unstructured proteins, such as α-synuclein and α_s-casein, are heat-resistant since the proteins have a similar unfolded conformation regardless of the temperature and their unfolded conformation is stable at high temperatures as well as at room temperature (*11*). The thermal behavior of α-synuclein and GST protein was initially compared using a qualitative heat-induced protein aggregation assay. Each protein was boiled in a boiling water bath, and the protein solution was centrifuged to remove the precipitates. Subsequently, the supernatant was analyzed on an SDS−polyacrylamide gel (Figure 2). As expected, α-synuclein did not precipitate upon

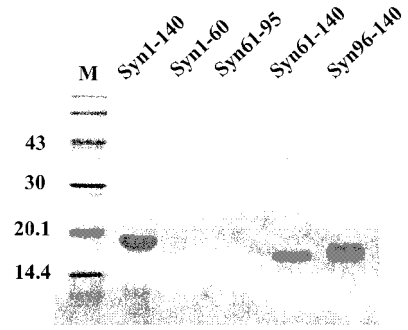

FIGURE 3: Thermal behavior of α-synuclein deletion mutants. The GST−α-synuclein fusion proteins were treated with thrombin, and the cleaved products were boiled in a boiling water bath. The protein solutions were centrifuged, and the supernatants were analyzed on a 12% SDS−polyacrylamide gel. Syn96−140 appeared to run slower than Syn61−140 on SDS−PAGE as had been previously observed (*56*).

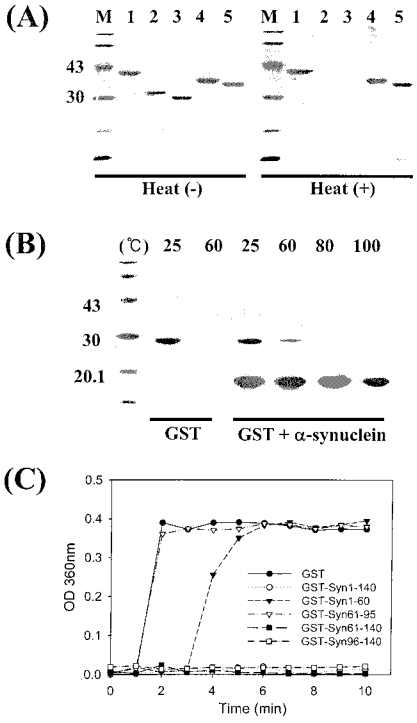

FIGURE 4: Thermal behavior of GST−α-synuclein fusion proteins. **(A)** SDS−PAGE analysis of the GST−α-synucelin fusion proteins before (left) and after (right) boiling: lane 1, GST−Syn1−140; lane 2, GST−Syn1−60; lane 3, GST−Syn61−95; lane 4, GST−Syn61−140; and lane 5, GST−Syn96−140. (B) α-Synuclein did not protect GST from heat-induced aggregation when the two proteins were mixed and incubated in a boiling water bath. α-Synuclein and GST were mixed (2:1 w/w ratio) and incubated at the indicated temperatures for 10 min. After centrifugation, supernatants were analyzed on an SDS gel. (C) Heat-induced aggregation of the GST−α-synucelin fusion proteins. Heat-induced aggregation of the GST and the GST−synuclein fusion proteins was quantitatively assessed by monitoring the light scattering (OD_{360}) as a function of time at 65 °C.

heat treatment, whereas the GST protein did. This indicates that the GST protein is a typical example of a heat-labile protein. The results were reproducible regardless of the pH (5−8.5) and salt concentration (0−0.5 M) of the buffer solution, and the protein concentration (0.1−5 mg/mL).

Thermal Behavior of α-Synuclein Deletion Mutants. The thermal stability of the α-synuclein deletion mutants obtained by thrombin digestion of GST−synuclein fusion proteins was next examined. The GST−α-synuclein fusion proteins were treated with thrombin, and the cleaved products were boiled in a boiling water bath. The protein solutions were centrifuged, and the supernatants were analyzed on an SDS−polyacrylamide gel. As shown in Figure 3, the wild type (Syn1−140) and two deletion mutants containing the acidic tail (Syn61−140 and Syn96−140) were found to be heat-resistant. In contrast, the N-terminal part of α-synuclein (Syn1−60) and the NAC peptide (Syn61−95) appeared to precipitate upon heat treatment. Interestingly, only the deletion mutants containing the C-terminal acidic tail were heat-resistant, indicating that the C-terminal acidic tail is responsible for the heat resistance. α-Synuclein has the ability to aggregate over time, or upon incubating at 37 °C to form amyloid fibril (reviewed in refs *18*−*20* and *30*). Consistent with our data, previous studies have shown that C-terminally truncated α-synuclein proteins and the NAC peptide assembled into filaments much more readily than the wild-type protein (*31*−*34*). Overall, it appears to be likely that C-terminally truncated α-synuclein mutant proteins are less stable at room temperature and higher temperatures than both the wild type and mutant proteins containing the C-terminal acidic tail.

Thermal Behavior of GST−Synuclein Fusion Proteins. The thermal behavior of GST−synuclein fusion proteins was similarly investigated. As shown in Figure 4A, GST−Syn1−140, GST−Syn61−140, and GST−Syn96−140 fusion proteins did not precipitate regardless of the heat treatment, indicating that these proteins are heat-resistant; on the other hand, GST−Syn1−60 and GST−Syn61−95 fusion proteins appeared to be heat-labile, and the proteins had completely precipitated upon heat treatment. Unlike the cases of GST−α-synuclein fusion proteins, α-synuclein did not protect GST from heat-induced aggregation when the two proteins were mixed and incubated in a boiling water bath (Figure 4B). Consistent with previous reports (*21*, *35*), however, α-synuclein appeared to have chaperone-like activity to protect GST from heat-induced aggregation at a relatively mild temperature (60 °C). These results suggest that the covalent bond between GST and α-synuclein is critical for extreme thermosolubility of the fusion protein.

The heat-induced aggregation of the GST−synuclein fusion proteins was quantitatively assessed by measuring the turbidity at 65 °C as a function of time. As shown in Figure 4C, the OD_{360} of the GST protein drastically increased 2 min after heat treatment, and most of the protein had aggregated by 3 min. The GST−Syn61−95 fusion protein behaved like the GST protein, and resulted in complete aggregation. The GST−Syn1−60 fusion protein also resulted in complete aggregation after heat treatment, although aggregation of this protein was relatively delayed. Consistent with the results depicted in Figure 4A, there was no evidence of any protein aggregation for GST−Syn1−140, GST−Syn61−140, and GST−Syn96−140 fusion proteins even after heat treatment for 30 min. Interestingly, these three heat-resistant GST−synuclein fusion proteins all contain the acidic tail of α-synuclein. This suggests that a heat-labile protein can be transformed into a heat-resistant protein by introducing the α-synuclein acidic tail.

Previously, many of the heat-resistant proteins from Jurkat T cell lysates and human serum were reported to be highly acidic proteins (*11*), suggesting that the pI value may be

Table 1: Isoelectric Point (pI) and Hydropathy Values of α-Synuclein and Its Deletion Mutants, as Well as GST and GST−Synuclein Fusion Proteins

protein	thermal behavior	pI value[a]	hydropathy value[b]
α-synuclein	HR[c]	4.67	−0.403
Syn1−60	HL[d]	9.52	−0.188
Syn61−95	HL	4.53	0.726
Syn61−140	HR	3.85	−0.564
Syn96−140	HR	3.76	−1.567
GST	HL	6.18	−0.390
GST−Syn1−140	HR	5.25	−0.378
GST−Syn1−60	HL	7.64	−0.349
GST−Syn61−95	HL	6.01	−0.244
GST−Syn61−140	HR	4.95	−0.435
GST−Syn96−140	HR	4.85	−0.560

[a] The pI values were calculated by using the ProtParam program (www.expasy.ch). [b] The hydropathy values were calculated by using the ProtParam program. [c] HR, heat-resistant. [d] HL, heat-labile.

related to the protein's heat resistance. The solubility of proteins may play an important role in determining the heat resistance, since highly charged proteins would be more soluble even at higher temperatures. To confirm this hypothesis, the pI and hydropathy values of α-synuclein with its deletion mutants and those of GST and GST−synuclein fusion proteins were compared (Table 1). Table 1 clearly shows that heat-resistant proteins, such as α-synuclein, Syn61−140, Syn96−140, GST−Syn1−140, GST−Syn61−140, and GST−Syn96−140, have abnormally low pI and hydropathy values, whereas the heat-labile proteins (Syn1−60, GST, GST−Syn1−60, and GST−Syn61−95) with the exception of Syn61−95 have much higher values. Interestingly, a heat-labile peptide Syn61−95 has a very low pI value, but it has an extremely high hydropathy value (Table 1). Therefore, it is possible that a highly charged protein with a low hydropathy value possesses an advantage in resisting heat-induced protein aggregation.

Effect of Divalent Cation Binding. Some divalent cations, such as Cu^{2+} and Ca^{2+}, are known to bind specifically to the C-terminal acidic tail of α-synuclein with a dissociation constant in the micromolar range (*24*, *36*). Zn^{2+} and other metal ions also appear to bind specifically to α-synuclein, although the binding sites are yet to be identified (*22*, *24*, *25*). Since the C-terminal acidic tail of α-synuclein is important for protein heat resistance, the effect of the divalent cation binding on the heat-induced aggregation of GST−synuclein fusion proteins containing the C-terminal acidic tail was investigated. Figure 5 shows that low concentrations of the divalent cations do not affect the heat-induced aggregation of the fusion proteins. However, high concentrations significantly increased the level of protein aggregation, although the fusion proteins do not result in complete precipitation. Particularly, Zn^{2+} appeared to be most effective for enhancing the heat-induced protein aggregation. If one considers the fact that the dissociation constants between α-synuclein and the divalent cations are considerably low (*24*, *36*) and that most proteins are affected by a high concentration of metal ions, the results suggest that the specific binding of the divalent cations at the C-terminal acidic tail of α-synuclein does not affect the thermal behavior of the fusion proteins. However, nonspecific binding of the metal ions at a high concentration appears to induce more protein aggregation during heat treatment.

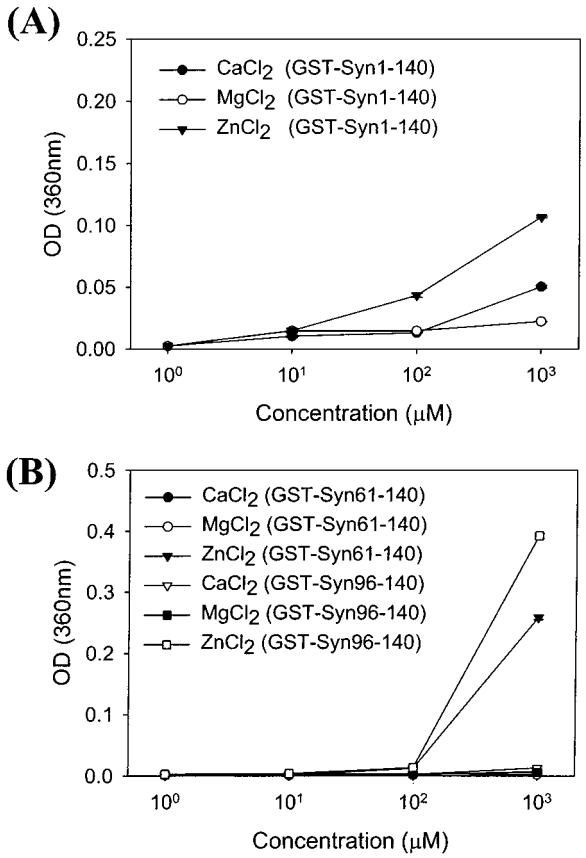

FIGURE 5: Effect of divalent cations on the thermal behavior of the GST−synuclein fusion proteins. Heat-induced aggregation of the GST−Syn1−140 (A) and GST−Syn61−140 and GST−Syn96−140 (B) fusion proteins at 65 °C was assessed as described in the legend of Figure 4, in the presence of the indicated cation concentrations.

Enzyme Activity of GST−Synuclein Fusion Proteins after Heat Treatment. Unlike the wild-type GST protein, GST fusion proteins containing the acidic tail of α-synuclein were shown to be heat-resistant. This suggests that the heat-labile protein could be transformed into a heat-resistant protein simply by introducing the acidic tail of α-synuclein. We next investigated whether the heat-resistant GST fusion proteins could keep the enzymatic activity after heat treatment. The GST and GST−synuclein fusion proteins were boiled in a water bath for 10 min and cooled in the air to room temperature. The catalytic activities of these heat-treated proteins were then compared. As shown in Figure 6A, all the GST and GST fusion proteins completely lost their enzymatic activity under these conditions. Subsequently, the thermostability of GST and the GST−Syn96−140 fusion protein was quantitatively measured by thermal inactivation curves (Figure 6b), which were used to determine the T_{50} values, the temperatures at which 50% of initial enzymatic activity is lost after heat treatment. As shown in Figure 6B, the T_{50} of the GST−Syn96−140 fusion protein is ∼2 °C higher than that of GST. Interestingly, the thermal inactivation of GST is well-correlated with the level of thermal aggregation of the protein. This suggests that the introduced acidic tail is able to protect the enzyme from the thermal inactivation by preventing the thermal aggregation of the fusion protein.

Heat-Induced Secondary Structural Changes of the GST−Syn96−140 Fusion Protein. Previously, heat-induced sec-

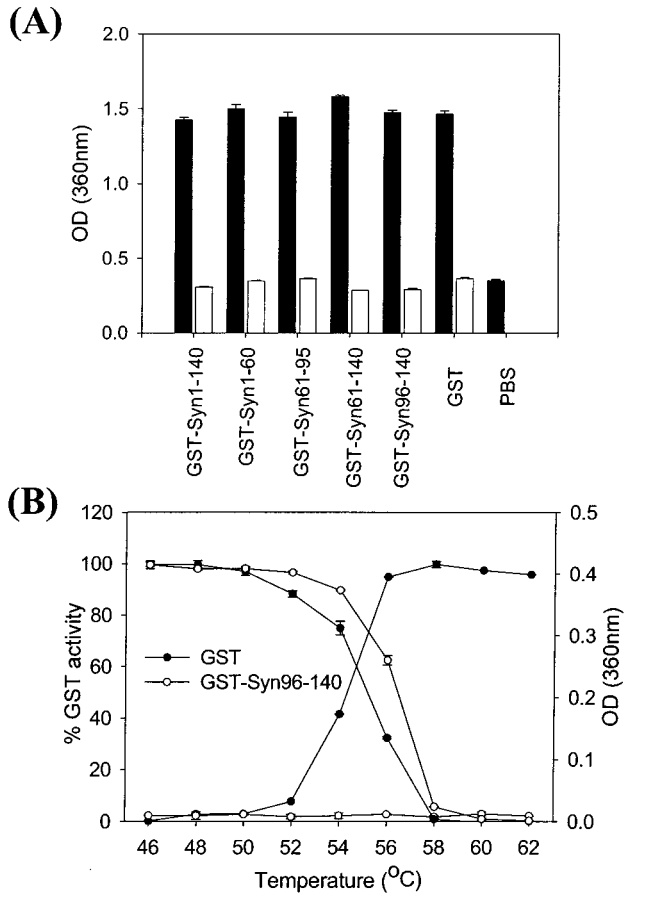

FIGURE 6: GST activity of the GST−synuclein fusion proteins after heat treatment. (A) GST and the GST fusion proteins completely lost their enzymatic activity after boiling in a water bath for 10 min (white bars). (B) Thermal inactivation and aggregation of GST and the GST−Syn96−140 fusion protein. Activity is expressed as a percentage of initial activity (left). Values are the means of three independent experiments with the standard deviations shown as bars. Heat-induced aggregation was quantitatively assessed by monitoring the light scattering (OD_{360}) as a function of temperature (right). The protein samples were incubated for 5 min at the indicated temperatures.

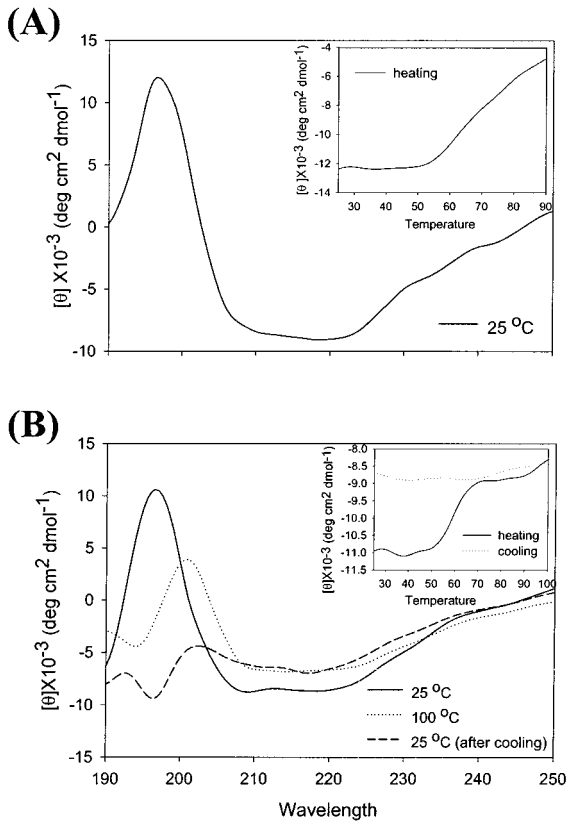

FIGURE 7: Far-UV CD spectra and melting curves of (A) GST and (B) the GST−Syn96−140 fusion protein. Far-UV CD spectra measured at 20 and 100 °C are drawn as solid lines and dotted lines, respectively. The dashed lines represent the spectra measured just after cooling of the protein solution from 100 to 20 °C. The insets show the mean molar ellipticity per residue of each protein at 222 nm as a function of temperature. The solid and dotted lines represent temperature scans from 20 to 100 °C (heating mode) and from 100 to 20 °C (cooling mode), respectively.

ondary structural changes of α-synuclein assessed by CD analysis were reported (Figure 3A in ref *11*). The CD spectrum of α-synuclein indicated that the protein almost completely lacks secondary structural elements. The CD spectrum of α-synuclein at 100 °C was slightly different from that at 25 °C, but it also represented the characteristics of random coiled polypeptides. Consistent with these results, a linear temperature dependence of the CD signal, often seen with unfolded peptides, was observed.

The CD spectrum of GST at 25 °C (Figure 7A) indicates that the protein contains well-ordered secondary structural elements. However, at 100 °C, the far-UV CD spectrum was greatly diminished in magnitude due to protein precipitation (data not shown). The temperature-induced changes in the ellipticity of GST at 222 nm indicate that the T_m of GST is approximately 70 °C. The GST had completely precipitated at 100 °C, and the repetition temperature scan showed the absence of any CD signal at 222 nm, indicating that GST had irreversibly precipitated (data not shown). These results confirm that the GST protein is a typical heat-labile protein that unfolds and precipitates as the temperature is increased.

The far-UV CD spectra of the GST−Syn96−140 fusion protein are shown in Figure 7B. The far-UV CD spectrum of the GST−Syn96−140 fusion protein at room temperature (solid line) indicates that the protein contains well-ordered secondary structural elements. The CD spectrum showed a decrease in the level of these elements at 100 °C, but the overall shape was unchanged (dotted line), suggesting that heating does not lead to complete unfolding. Interestingly, a new absorption band at 195 nm appears, which is characteristic of random coiled polypeptides. After the protein had cooled, the far-UV CD spectrum (dashed line) remained distinguishable from the initial one, suggesting that the conformation of the GST−Syn96−140 fusion protein may be irreversibly changed. The CD spectrum of the heat-treated GST−Syn96−140 fusion protein at room temperature rather resembles that obtained at 100 °C, and indicates that the protein consists of two distinct domains: one with regular secondary structural elements and the other with a random coil-like conformation. To confirm the conformational changes induced by heating, the GST−Syn96−140 melting curves were measured as a function of temperature. The temperature-induced changes in ellipticity at 222 nm are presented in the inset of Figure 7B. Interestingly, the heat-induced unfolding of the GST−Syn96−140 fusion protein appeared to take place in two stages (solid line). The transition midpoint appeared at 62 °C for the first transition

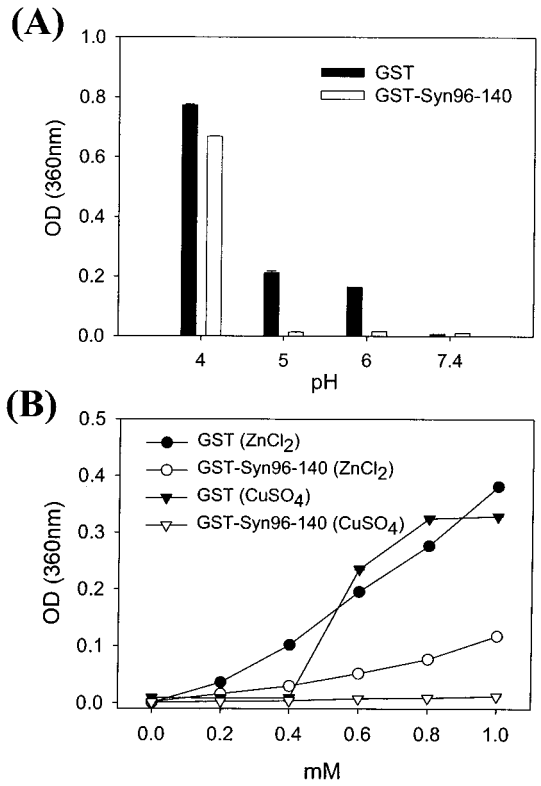

FIGURE 8: Effects of the acidic tail on stress-induced protein aggregation. (A) pH-induced aggregation of GST and the GST−Syn96−140 fusion protein at room temperature. (B) Metal-induced aggregation of GST and the GST−Syn96−140 fusion protein at room temperature. Protein aggregation was monitored by light scattering analysis at 360 nm.

and at 95 °C for the second. As expected, the temperature course for the GST−Syn96−140 fusion protein appeared to be irreversible (dotted line).

GST is a heat-labile protein, while the GST−Syn96−140 fusion protein is a heat-resistant protein. To compare the stability of the two proteins, it would be useful to determine the melting temperatures (T_m) of both proteins. However, it is difficult to compare the T_m values of the GST−Syn96−140 fusion protein and GST directly since the proteins contain a different number of peptide domains. Interestingly, the T_m value of the GST−Syn96−140 fusion protein (62 °C for the first transition) appeared to be slightly lower than that of GST (70 °C). Since the T_m of a given protein is related to the change in the free energy between the native and thermally denatured state of a protein, the T_m has been used as a thermodynamic parameter of the conformational stability of the protein. Therefore, it is highly likely that introducing the acidic tail to the C-terminus of GST is favorable for protein solubility and consequently for heat resistance, but unfavorable for the intrinsic stability of the protein.

pH- and Metal-Induced Protein Aggregation. The pH-induced aggregation of GST and the GST−Syn96−140 fusion protein was investigated by measuring the turbidity at 25 °C as a function of pH. As shown in Figure 8A, the OD_{360} of the GST protein steadily increased from pH 7.4 to 5.0 and reached a maximum value at pH 4.0, whereas the OD_{360} of the GST−Syn96−140 fusion protein was unchanged until pH 5.0, but drastically increased at pH 4.0 perhaps due to the neutralization of the acidic tail. This suggests that the C-terminal acidic tail is able to protect GST from pH-induced aggregation, though the protection effect is not sufficient under very acidic conditions. The C-terminal acidic tail also appeared to protect GST from metal-induced aggregation (Figure 8B). The OD_{360} of the GST protein steadily increased when it was treated with 0.2−1.0 mM Zn^{2+}, while the OD_{360} of the GST−Syn96−140 fusion protein was always much lower than that of GST. In particular, Cu^{2+}-induced protein aggregation was completely blocked by the acidic tail. These results indicate that the C-terminal acidic tail can also protect GST from metal-induced aggregation.

DISCUSSION

α-Synuclein is a well-known heat-resistant protein that does not aggregate when treated with heat, whereas GST is a heat-labile protein that easily precipitates under thermal stress. In this study, we have shown that the C-terminal acidic tail of α-synuclein (residues 96−140) renders the protein heat-resistant. Furthermore, we have demonstrated that the GST−α-synuclein fusion protein is also heat-resistant. Like the α-synuclein, the fusion protein appears to be extremely thermosoluble even when it is boiled in a water bath. Using a series of GST−synuclein deletion mutants, we have shown that the C-terminal acidic tail of α-synuclein also plays a critical role in conferring heat resistance of the fusion proteins. Interestingly, the acidic tail turns out to protect the fusion protein from the loss of activity after heat treatment; a significant increase in thermostability has been observed from the GST−Syn96−140 fusion protein with a 2 °C increase in the T_{50} value. However, a systematic investigation of the heat-induced secondary structural changes of α-synuclein, GST, and the GST−α-synuclein fusion protein reveals that the fusion protein is irreversibly denatured by heat treatment with a slightly lowered T_m value (∼8 °C). Therefore, introducing an acidic tail into the GST protein appears to be favorable for thermosolubility and thermostability, but unfavorable for the intrinsic stability of the protein. Interestingly, the GST protein fused with the acidic tail appears to be more resistant to the pH- and metal-induced protein aggregation, suggesting that the acidic tail increases the virtual stability of the protein by protecting it from environmental stresses.

The C-terminal acidic tails of the synuclein family members are very diverse in size as well as in sequence (reviewed in refs *18*−*21*). When the fact that the N-terminal amphipathic region is strictly conserved among the synuclein family members from *Torpedo* to humans is considered, the C-terminal acidic tail may be responsible for the specificity of each synuclein protein. In this study, the C-terminal acidic tail of α-synuclein appears to play a critical role in conferring heat resistance to the GST fusion protein as well as to the synuclein protein itself. Presumably, the abolishment of heat-induced protein aggregation in α-synuclein and the GST−synuclein fusion proteins at high temperatures results from the facts that the acidic tail increases the solubility of protein by increasing the hydrophilicity of the protein and that the acidic tail makes the intermolecular interaction unfavorable by repulsion between negatively charged residues. This idea is supported by the observation that α-synuclein with a truncated C-terminal region and the NAC peptide lacking the C-terminal acidic tail are found to aggregate faster than the full-length α-synuclein under the same conditions (*31*−

34). It is well-documented that the solubility of a protein is approximately proportional to the square of the net charge on the protein (*37*). In fact, introducing the acidic tail significantly decreases the pI and hydropathy values of the fusion proteins (Table 1). Given that all the synuclein proteins are extremely heat-resistant, all the acidic tails from the synuclein proteins may increase the solubility of the fusion proteins and consequently could make the proteins heat-resistant.

Introducing the C-terminal acidic tail of α-synuclein appears to transform a heat-labile protein into a heat-resistant protein. Analysis of the heat-induced secondary structural changes of GST and the GST−Syn96−140 fusion protein indicates that the T_m of the fusion protein is ∼8 °C lower than that of GST itself. This suggests that the acidic tail of the fusion protein destabilizes the GST domain in terms of the T_m (Figure 7). It is well-documented that nonspecific repulsions, which arise when a protein is highly charged, affect protein stability (reviewed in ref *38*). As the net charge on the native protein is increased, the increasing level of charge repulsion destabilizes the folded protein. It has been also reported that the exposed hydrophilic residues themselves are unfavorable for protein stability, since the region becomes more flexible (*39*−*41*). However, it is somewhat inappropriate to directly compare the T_m value of a one-domain protein (GST) with that of a two-domain protein (GST−synuclein). The stability of each domain in a multi-domain protein is usually affected by the stability of the other domain. Introducing a more stable domain stabilizes the other domain, but a less stable domain could destabilize the other domain (*42*−*45*).

The acidic tail appears to protect the fusion protein from thermal inactivation to a considerable extent. The T_{50} values of GST and the GST−Syn96−140 fusion protein are 55.5 and 57.5 °C, respectively, suggesting that the acidic tail increases the thermostability of the fusion protein. Presumably, a significant increase in thermostability observed from the GST−Syn96−140 fusion protein results from the fact that the acidic tail protects the protein from heat-induced aggregation. As shown in Figure 6B, GST aggregates from 52 °C, which is much lower than the T_m (70 °C). Interestingly, loss of GST activity is very well correlated with the aggregation of the protein that occurs prior to complete unfolding. On the other hand, the GST−Syn96−140 fusion protein does not aggregate, but it unfolds at high temperatures with a T_m of 62 °C. For the GST−Syn96−140 fusion protein, loss of activity appears to result from the unfolding of the protein. The acidic tail also appears to protect the fusion protein from pH- and metal-induced aggregation. Therefore, it is likely that the introduced acidic tail increases the virtual stability by protecting the protein from stress-induced aggregation, although the effect on thermostability is limited due to the decrease of the T_m (from 70 to 62 °C). The destabilizing effect of the introduced acidic tail on protein stability could be minimized by shortening the size of the acidic tail.

Most of the small molecular chaperone proteins, such as HSP25, α-crystalline, tubulin, etc., contain a unique flexible hydrophilic tail at the C-terminus, and this hydrophilic tail is important for their molecular chaperone function (*46*−*49*). α-, β-, and γ-synuclein proteins also have chaperone-like activity, and the C-terminal acidic tail of α-synuclein plays a critical role in this chaperone-like activity (*22*, *35*). Deleting the acidic tail abolishes the chaperone-like activity of α-synuclein (*35*). Therefore, it is also possible to speculate that the heat resistance of α-synuclein and the GST−synuclein fusion protein might originate from their self-chaperoning activity. However, the possibility is less likely since α-synuclein and the GST−synuclein fusion protein never precipitate even when they are boiled in a water bath for a long time, and these proteins do not form oligomers when treated with heat. These phenomena are quite distinct from those of a molecular chaperone and a substrate protein system, where the chaperoning effect is limited to mild temperature ranges and the stable complex of a chaperone−substrate protein is usually observed when the mixtures are treated with heat (*50*−*52*). In fact, our data clearly demonstrate that α-synuclein cannot protect GST from heat-induced aggregation when the two proteins are mixed and incubated at higher temperatures (Figure 4B). Thus, it is highly likely that the extreme heat resistance of α-synuclein and the GST−α-synuclein fusion protein is their intrinsic property.

α-Synuclein has the potential to bind several divalent cations and metal ions, including Fe^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}, and Ca^{2+} (*22*, *24*−*26*, *36*). Metal ions (Fe^{2+}, Al^{3+}, Zn^{2+}, and Cu^{2+}) bind to α-synuclein and induce self-oligomerization of the protein. Cu^{2+} and Ca^{2+} are known to bind specifically to the C-terminal acidic tail with a dissociation constant of 59 μM and an IC_{50} of 300 μM, respectively (*24*, *36*). However, the binding sites and binding constants of Fe^{2+}, Al^{2+}, and Zn^{2+} have yet to be determined. In this study, divalent cation binding does not appear to affect the thermal behavior of both α-synuclein and the GST−α-synuclein fusion proteins, although nonspecific binding at high cation concentrations appears to induce a certain amount of protein aggregation. Previous studies have shown that Zn^{2+} binding at a high concentration (∼5 mM) completely abolishes the chaperone-like activity of α-synuclein, presumably due to the conformational change of α-synuclein (*22*). However, the data presented here suggest that the chaperone-like activity also would not be affected by specific binding of the cations at low concentrations.

The NAC peptide (residues 61−95) has been identified as the second major component of the senile plaques in the brains of Alzheimer's disease patients (*14*), and C-terminally truncated α-synuclein has been found in the Lewy bodies in the brains of Parkinson's disease patients (*53*, *54*). This suggests that abnormal digestion of α-synuclein under certain conditions may be involved in the pathology of the neurodegenerative diseases. Earlier works have shown that α-synuclein is extremely sensitive to most proteases (*16*). Interestingly, a series of α-synuclein deletion mutants appear to behave differently after thermal stress (Figure 3). Syn1−60 and Syn61−95 easily precipitate during heat treatment, whereas Syn61−140 and Syn96−140 are extremely heat-resistant. Earlier works have also demonstrated that the NAC peptide is amyloidogenic by itself and stimulates A_β aggregation (*33*, *34*), and the C-terminally truncated α-synuclein proteins assembled into filaments much more readily than the wild-type protein (*32*). Overall, it is highly likely that some types of α-synuclein protease digestion result in the formation of unstable peptide fragments, which may confer pathologic protein aggregation.

In summary, we have demonstrated that the introduction of the acidic tail of α-synuclein into a heat-labile protein protects the protein from environmental stresses, such as heat, pH, and metal ions. Consequently, the acidic tail greatly increases the thermosolubility of the fusion protein and significantly improves the thermostability. Overall, our data suggest that the acidic tail contributes to the virtual stability of the protein, although it does not appear to increase its intrinsic stability. Introducing the acidic tail also contributes to the protein solubility since it will greatly increase the hydrophilicity of the protein and make intermolecular interactions unfavorable through electrostatic repulsion. Therefore, the acidic tail of α-synuclein can be utilized to increase protein solubility and to protect the protein from environmental stresses. Many biologically or medically important proteins that have solubility problems or stress-induced aggregation problems might be saved by introducing the acidic tail of α-synuclein. To the best of our knowledge, this is the first report showing that an acidic tail can be utilized to greatly increase the solubility and virtual stability of target proteins. The polyhistidine tag (His tag) originally developed to facilitate purification (reviewed in ref *55*) has also been implicated in increasing the solubility of target proteins to a certain degree, although the effect and its mechanism have not been intensively analyzed. Since the His tag is mildly charged under physiological conditions (pK_a of His ≈ 6.2), its solubilizing effect is expected to be much lower in magnitude than that of the acidic tail of α-synuclein which contains 15 Glu/Asp residues (pK_a of Glu/Asp ≈ 4.5).

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REFERENCES

1. Bull, H. B., and Breese, K. (1973) *Arch. Biochem. Biophys. 156*, 604−612.
2. Jaenicke, R., and Böhm, G. (1998) *Curr. Opin. Struct. Biol. 8*, 738−748.
3. Rees, D. C., and Adams, M. W. W. (1995) *Structure 3*, 251−254.
4. Adams, M. W. W. (1993) *Annu. Rev. Microbiol. 47*, 627−658.
5. Cavagnero, S., Zhou, Z. H., Adams, M. W. W., and Chan, S. I. (1995) *Biochemistry 34*, 9865−9873.
6. Klump, K. H., Adams, M. W. W., and Robb, F. T. (1994) *Pure Appl. Chem. 66*, 485−489.
7. Klump, H., DiRuggiero, J., Kerssel, M., Park, J.-B., Adams, M. W. W., and Robb, F. T. (1992) *J. Biol. Chem. 267*, 22681−22685.
8. Wright, P. E., and Dyson, H. J. (1999) *J. Mol. Biol. 293*, 321−331.
9. Plaxco, K. W., and Gross, M. (1997) *Nature 386*, 657−658.
10. Uversky, V. N., Gillespie, J. R., and Fink, A. L. (2000) *Proteins 41*, 415−427.
11. Kim, T. D., Ryu, H. J., Cho, H. I., Yang, C. H., and Kim, J. (2000) *Biochemistry 39*, 14839−14846.
12. Uversky, V. N., Li, J., and Fink, A. L. (2001) *J. Biol. Chem. 276*, 10737−10744.
13. Jakes, R., Spillantini, M. G., and Goedert, M. (1994) *FEBS Lett. 345*, 27−32.
14. Ueda, K., Fukushima, H., Masliah, E., Xia, Y., Iwai, A., Yoshimoto, M., Otero, D. A. C., Kondo, J., Ihara, Y., and Saitoh, T. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11282−11286.
15. Eliezer, D., Kutluay, E., Bussell, R., and Browne, G. (2001) *J. Mol. Biol. 307*, 1061−1073.
16. Kim, J. (1997) *Mol. Cells 7*, 78−83.
17. Weinreb, P. H., Weiguo, Z., Poon, A. W., Conway, K. A., and Lansbury, P. T. (1996) *Biochemistry 35*, 13709−13715.
18. Lücking, C. B., and Brice, A. (2000) *Cell. Mol. Life Sci. 57*, 1894−1908.
19. Iwai, A. (2000) *Biochim. Biophys. Acta 1502*, 95−109.
20. Hashimoto, M., and Masliah, E. (1999) *Brain Pathol. 9*, 707−720.
21. Lavedan, C. (1998) The synuclein family, *Genome Res. 8*, 871−880.
22. Kim, T. D., Paik, S. R., Yang, C. H., and Kim, J. (2000) *Protein Sci. 9*, 2489−2496.
23. Davidson, W. S., Jonas, A., Clayton, D. F., and George, J. M. (1998) *J. Biol. Chem. 273*, 9443−9449.
24. Paik, S. R., Shin, H. J., Lee, J. H., Chang, C. S., and Kim, J. (1999) *Biochem. J. 340*, 821−828.
25. Uversky, V. N., Li, J., and Fink, A. L. (2001) *J. Biol. Chem. 276*, 44284−44296.
26. Paik, S. R., Lee, J.-H., Kim, D.-H., Chang, C.-S., and Kim, J. (1997) *Arch. Biochem. Biophys. 344*, 325−334.
27. Lee, G. J., and Vierling, E. (1998) *Methods Enzymol. 290*, 350−365.
28. Horwitz, J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10449−10453.
29. Habig, W. H., Pabst, M. J., and Jakoby, W. B. (1974) *J. Biol. Chem. 249*, 7130−7139.
30. Rajagopalan, S., and Andersen, J. K. (2001) *Mech. Ageing Dev. 122*, 1499−1510.
31. Serpell, L. C., Berriman, J., Jakes, R., Goedert, M., and Crowther, R. A. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 4897−4902.
32. Crowther, R. A., Jakes, R., Spillantini, M. G., and Goedert, M. (1998) *FEBS Lett. 436*, 309−312.
33. Han, H., Weinreb, P. H., and Lansbury, P. T., Jr. (1995) *Chem. Biol. 2*, 163−169.
34. Iwai, A., Yoshimoto, M., Masliah, E., and Saitoh, T. (1995) *Biochemistry 34*, 10139−10145.
35. Souza, J. M., Giasson, B. I., Lee, V. M. Y., and Ischiropoulos, H. (2000) *FEBS Lett. 474*, 116−119.
36. Nielsen, M. S., Vorum, H., Lindersson, E., and Jensen, P. H. (2001) *J. Biol. Chem. 276*, 22680−22684.
37. Tanford, C. (1961) *Physical chemistry of macromolecules*, John Wiley and Sons, Inc., New York.
38. Dill, K. A. (1990) *Biochemistry 29*, 7133−7155.
39. Ponomarev, M. A., Furch, M., Levitsky, D. I., and Manstein, D. J. (2000) *Biochemistry 39*, 4527−4532.
40. Spector, S., Wang, M., Carp, S. A., Robblee, J., Hendsch, Z. S., Fairman, R., Tider, B., and Raleigh, D. P. (2000) *Biochemistry 39*, 872−879.
41. Russell, R. J. M., Hough, D. W., Danson, M. J., and Taylor, G. L. (1994) *Structure 2*, 1157−1167.
42. Deyrup, A. T., Krishnan, S., Singh, B., and Schwartz, N. B. (1999) *J. Biol. Chem. 274*, 10751−10757.
43. Zaiss, K., and Jaenicke, R. (1999) *Biochemistry 38*, 4633−4639.
44. Beaucamp, N., Hofmann, A., Kellerer, B., and Jaenicke, R. (1997) *Protein Sci. 6*, 2159−2165.
45. Yablonski, M., Pasek, D., Han, B., Jones, M., and Traut, T. (1996) *J. Biol. Chem. 271*, 10704−10708.
46. Guha, S., Manna, T. K., Das, K. P., and Bhattacharyya, B. (1998) *J. Biol. Chem. 273*, 30077−30080.
47. Smulders, R. H. P. H., Carver, J. A., Lindner, R. A., van Boekel, M. A. M., Bloemendal, H., and de Jong, W. W. (1996) *J. Biol. Chem. 271*, 29060−29066.
48. Carver, J. A., Esposito, G., Schwedersky, G., and Gaestel, M. (1995) *FEBS Lett. 369*, 305−310.
49. Das, K. P., Petrash, J. M., and Surewicz, W. K. (1996) *J. Biol. Chem. 275*, 10449−10452.
50. Farahbakhsh, Z. T., Huang, Q. L., Ding, L. L., Altenbach, C., Steinhoff, H. J., Horwitz, J., and Hubbel, W. (1995) *Biochemistry 34*, 509−516.

51. Marini, I., Moschini, R., Corso, A. D., and Mura, U. (2000) *J. Biol. Chem. 275*, 32559−32565.
52. Rao, P. V., Horwitz, J., and Zigler, J. S. (1993) *Biochem. Biophys. Res. Commun. 190*, 786−793.
53. Baba, M., Nakajo, S., Tu, P. H., Nakaya, K., Lee, V. M. Y., Trojanowski, J. Q., and Iwatsubo, T. (1998) *Am. J. Pathol. 152*, 879−884.
54. Gai, W. P., Power, J. H. T., Blumbergs, P. C., Culvenor, J. G., and Jensen, P. H. (1999) *J. Neurochem. 73*, 2093−2100.
55. Crowe, J., Dobeli, H., Gentz, R., Hochuli, E., Stuber, D., and Henco, K. (1994) *Methods Mol. Biol. 31*, 371−387.
56. Jensen, P. H., Hager, H., Nielsen, M. S., Hojrup, P., Gliemann, J., and Jakes, R. (1999) *J. Biol. Chem. 274*, 25481−25489.

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