Protein Engineering, Design & Selection vol. 17 no. 3 pp. 251–260, 2004
Published online April 5, 2004 DOI: 10.1093/protein/gzh029

Effects of novel peptides derived from the acidic tail of synuclein (ATS) on the aggregation and stability of fusion proteins

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The acidic tail of α-synuclein (ATSα) has been shown to protect the glutathione *S*-transferase (GST)–ATSα fusion protein from environmental stresses, such as heat, pH and metal ions. In this study, we further demonstrated that the introduction of ATSα into other proteins, such as dehydrofolate reductase and adiponectin, renders the fusion proteins resistant to heat-induced aggregation and that the acidic tail of β- or γ-synuclein can also protect the fusion proteins from heat-induced aggregation. Interestingly, the heat resistance of GST–ATSα deletion mutants, which contain shorter peptides derived from the highly charged regions of ATSα, was approximately proportional to the number of added Glu/Asp residues. However, the negative charges in the ATSα-derived peptides appear insufficient to explain the extreme heat resistance of the fusion proteins, since polyglutamates appeared to be much less effective than the ATSα-derived peptides in conferring heat resistance on the fusion proteins. These results suggest that not only the negatively charged residues but also the specific amino acid sequence of ATSα play an important role in conferring extreme heat resistance on the fusion proteins. Furthermore, the heat-induced secondary structural changes and thermal inactivation curves of GST–ATSα deletion mutants indicated that the introduction of ATSα-derived peptides does not significantly affect the intrinsic stability of the fusion proteins.

Keywords: fusion protein/heat-resistant proteins/protein aggregation/protein stability/α-synuclein

Introduction

Protein aggregation is a complex phenomenon that can occur both *in vivo* and *in vitro*, usually resulting in the irreversible loss of the protein's biological activity (Cleland *et al*., 1993; Speed *et al*., 1995, 1996; Carpenter *et al*., 1999). In many cases, protein aggregation *in vivo* leads to protein deposition diseases. For example, it has been suggested that many neurological disorders, such as Alzheimer's disease, Parkinson's disease, Huntington's disease and prion disease, are caused by the abnormal aggregation and deposition of Aβ, α-synuclein, huntingtin and prion, respectively (Tan and Pepys 1994; Speed *et al*., 1996; Serpell *et al*., 1997; Cohen, 1999; Lansbury, 1999; Lucking and Brice, 2000; Ghiso and Frangione, 2002; Ross, 2002). Protein aggregation *in vitro*, which occurs even under conditions favoring the native state, leads to major economic and technical problems in the biotechnology and biopharmaceutical industries. In particular, the overexpression of some eukaryotic proteins in heterologous hosts results in the formation of inclusion bodies (Williams *et al*., 1982; Hlodan *et al*., 1991; Kane and Hartley, 1991; Jaenicke, 1995). These inclusion bodies have to undergo a refolding process to recover their biological activity. However, the process of renaturation is frequently difficult and the efficiency is very low, as a result of protein aggregation during the *in vitro* refolding processes. Moreover, the soluble recombinant proteins also aggregate frequently during the purification, storage and handling process in the presence of chemical, physical and thermal stresses (Manning *et al*., 1989; Cleland *et al*., 1993; Fink, 1998; Przybycien, 1998).

Protein aggregation is a major problem, particularly in the biomedical and biopharmaceutical fields (Cleland *et al*., 1993; Carpenter *et al*., 1999). In addition to eliminating or reducing the protein's therapeutic efficacy and shelf-life, the aggregation of parenterally administrated proteins can cause adverse patient reactions, such as an unwarranted immune response, hypersensitization or even anaphylactic shock (Moore *et al*., 1980; Robbins *et al*., 1987; Ratner *et al*., 1990; Thornton and Ballow, 1993; Braun *et al*., 1997). Therefore, it is essential that aggregate formation should be prevented during all stages of product handling. For this reason, many researchers have been trying to overcome protein aggregation problems (Manning *et al*., 1989; Carpenter *et al*., 1999; Talaga, 2001; Horwich, 2002; Schlieker *et al*., 2002). Usually, therapeutic proteins are protected against potential stresses by the addition of proper excipients or additives. In addition, chemical modification and site-directed mutagenesis of proteins are often adopted to produce a more stable form.

In a previous report, we demonstrated that the introduction of the C-terminal acidic tail of α-synuclein (ATSα) into a heat-labile protein, glutathione *S*-transferase (GST), protects the fusion protein from heat-induced aggregation (Park *et al*., 2002a). Furthermore, ATSα appears 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 (Park *et al*., 2002a). In an extension of this study, we systematically investigated the effects of novel peptides derived from the C-terminal acidic tails of synuclein family members (ATS) on the aggregation and stability of fusion proteins.

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 was obtained from Peptron (Taejon, Korea) and Ni-NTA resin from Invitrogen

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Table I. The oligonucleotide sequences and their coding peptides of ATSα deletion mutants, E5 and E10 (S, sense; AS, antisense)

Peptide name	Synthetic oligonucleotides		Coding peptides
Syn103–115	S	5′-gatccaatgaagaaggagccccacaggaaggcattctggaagattaag-3′	NEEGAPQEGILED
	AS	5′-aattcttaatcttccagaatgccttcctgtggggctccttcttcattg-3′	
Syn114–126	S	5′-gatccgaagatatgcctgtagatcctgacaatgaggcttatgaataag-3′	EDMPVDPDNEAYE
	AS	5′-aattcttattcataagcctcattgtcaggatctacaggcatatcttcg-3′	
Syn119–140	S	5′-gatccgatcctgacaatgaggcttatgaaatgccttctgaggaagggtatcaa-gactacgaacctgaagcctaag-3′	DPDNEAYEMPSEEGYQDYEPEA
	AS	5′-aattcttaggcttcaggttcgtagtcttgatacccttcctcagaaggcatttcataa-gcctcattgtcaggatcg-3′	
Syn130–140	S	5′-gatccgaggaagggtatcaagactacgaacctgaagcctaag-3′	EEGYQDYEPEA
	AS	5′-aattcttaggcttcaggttcgtagtcttgatacccttcctcg-3′	
E5	S	5′-gatccgaagaagaagaagaataag-3′	EEEEE
	AS	5′-aattcttattcttcttcttcttcg-3′	
E10	S	5′-gatccgaagaagaagaagaagaagaagaagaagaataag-3′	EEEEEEEEEE
	AS	5′-aattcttattcttcttcttcttcttcttcttcttcttcg-3′	
ATSα (Syn96–140)			KKDQLGKNEEGAPQEGILEDMPVD-PDNEAYEMPSEEGYQDYEPEA

(Carlsbad, CA). Leupeptin, pepstatin, phenylmethylsulfonyl fluoride (PMSF) and imidazole were purchased from Boehringer Manheim (Mannheim, Germany).

Preparation of dihydrofolate reductase (DHFR)–ATSα fusion protein

DHFR and DHFR–ATSα fusion protein were prepared as described previously (Park *et al*., 2002b).

Preparation of adiponectin and adiponectin–ATSα fusion protein

Adiponectin expression vector was produced by subcloning the globular domain of mouse adiponectin gene (residues 111–247) (Scherer *et al*., 1995) into the pRSETA vector. Adiponectin–ATSα fusion construct was produced by consecutively subcloning the globular domain of mouse adiponectin gene and the C-terminal acidic tail of α-synuclein (ATSα, residues 96–140) (Ueda *et al*., 1993; Jakes *et al*., 1994) into the pRSETA vector. Both constructs were verified by DNA sequencing.

The adiponectin and adiponectin–ATSα expression vectors were transformed into the *Escherichia coli* strain BL21 (DE3) for protein expression. One liter of bacteria carrying the recombinant plasmids was grown from a single colony at 25°C in LB medium containing 100 μg/ml ampicillin and 0.5% lactose. After overnight culture, bacteria were harvested by centrifugation. The recombinant adiponectin and adiponectin–ATSα fusion protein were purified by conventional column chromatographic techniques. The bacterial pellet was disrupted by sonication at 4°C in a lysis buffer containing 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.002% PMSF and 20 μg/ml leupeptin. The homogenate was then centrifuged for 10 min at 4°C at 10 000 r.p.m. The supernatant was loaded on to a DEAE-Sepharose CL 6B column, which was pre-equilibrated with 20 mM Tris–HCl buffer, pH 7.5, containing 0.1 M NaCl and 1 mM EDTA. The column was washed with the same buffer and the bound proteins were eluted from the column by a linear gradient from 0.1 to 0.4 M NaCl in 20 mM Tris–HCl buffer, pH 7.5. The eluted adiponectin fractions were pooled and concentrated and were further purified on an FPLC gel-filtration column equilibrated in 20 mM phosphate-buffered saline (PBS), pH 7.5.

Preparation of GST–ATSβ and GST–ATSγ fusion proteins

GST–ATSβ and GST–ATSγ fusion constructs were generated by subcloning the acidic tail of β-synuclein (ATSβ, residues 85–134) (Jakes *et al*., 1994) and the acidic tail of γ-synuclein (ATSγ, residues 96–127) (Ji *et al*., 1997), respectively, into pGEX vector (Pharmacia Biotech, Buckinghamshire, UK). The protein coding region of the ATSβ was amplified by PCR with the 5′-oligonucleotide primer AGCTAAGGATCCAAGA-GGGAGGAATTCC containing the underlined *Bam*HI restriction site and the 3′-oligonucleotide primer AAGTAA-CTCGAGCTACGCCTCTGGCTCATA containing the underlined *Xho*I restriction site. The protein coding region of the ATSγ was amplified by PCR with the 5′-oligonucleotide primer AAGAATGGATCCCGCAAGGAGGACTTGA containing the underlined *Bam*HI restriction site and the 3′-oligonucleotide primer AATAGCGAATTCCTAGTCTCCCCCACTCT containing the underlined *Eco*RI restriction site. The amplified DNAs were gel purified, digested with appropriate enzymes, then ligated into the pGEX vector that had been digested with appropriate restriction enzymes and gel purified. All constructs (pGST-ATSβ and pGST-ATSγ) were verified by DNA sequencing. The GST–synuclein fusion constructs, pGST-ATSβ and pGST-ATSγ, were transformed into the *E.coli* strain, BL21 (DE3) and the recombinant GST–synuclein fusion proteins (GST–ATSβ and GST–ATSγ) were purified by affinity chromatography using glutathione-Sepharose 4B beads. The GST–synuclein fusion proteins were further purified on an FPLC gel-filtration column pre-equilibrated with PBS, pH 7.4.

Deletion mutant forms of the GST–ATSα fusion protein

The DNAs encoding the parts of the acidic tail of α-synuclein (ATSα) were chemically synthesized (Table I). Using these synthetic cDNAs, a series of GST–ATSα deletion constructs were generated by ligating the parts of the ATSα gene into pGEX vector using the *Bam*HI and *Eco*RI restriction sites. GST–Syn103–115 contains 13 amino acids of the ATSα (residues 103–115), GST–Syn114–126 contains 13 amino acids of the ATSα (residues 114–126), GST–Syn119–140 contains 22 amino acids of the ATSα (residues 119–140) and GST–Syn130–140 contains 11 amino acids of the ATSα (residues 130–140) (Table I). All constructs

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(pGST-Syn103–115, pGST-Syn114–126, pGST-Syn119–140 and pGST-Syn130–140) were verified by DNA sequencing. The GST–ATSα deletion constructs, pGST-Syn103–115, pGST-Syn114–126, pGST-Syn119–140 and pGST-Syn130–140, were transformed into the *E.coli* strain BL21 (DE3) and the recombinant proteins were purified by affinity chromatography using glutathione-Sepharose 4B beads. The GST–ATSα deletion mutants were further purified on an FPLC gel-filtration column pre-equilibrated with PBS, pH 7.4.

Preparation of GST–polyE fusion proteins

The DNAs encoding the polyglutamates (pentamer and decamer, E5 and E10, respectively) were chemically synthesized (Table I). Using these synthetic cDNAs, GST–polyE fusion constructs were generated by ligating the annealed oligonucleotides into pGEX vector using the *Bam*HI and *Eco*RI restriction sites. All constructs were verified by DNA sequencing. The GST–polyE fusion proteins were purified by affinity chromatography using glutathione-Sepharose 4B beads as described above.

Heat-induced protein aggregation assay

The heat-induced aggregation of GST fusion proteins was qualitatively assayed by SDS–PAGE after heat treating the protein samples. Each protein in PBS (0.8 mg/ml) was heated in a boiling water-bath for 10 min and then cooled in the open air. The protein samples were centrifuged at 15 000 r.p.m. for 10 min and the supernatants were analyzed on a 12% SDS–polyacrylamide gel. The level of heat-induced aggregation of GST fusion proteins was also quantitatively measured by monitoring the apparent absorbance (scattering) at 360 nm as a function of time at 65°C (Horwitz, 1992; Lee and Vierling, 1998; Kim *et al*., 2000a; Uversky *et al*., 2001; Park *et al*., 2002a). Each protein was diluted to a final concentration of 0.2 mg/ml in the PBS buffer. The protein sample in the spectrophotometric cuvette was placed in a thermostatic cell holder and the apparent absorbance was monitored in a Beckman DU-650 spectrophotometer. Finally, the concentration-dependent protein aggregation of the GST fusion proteins was quantitatively assayed by monitoring their absorbance at 360 nm, while varying the concentration from 0.2 to 1.0 mg/ml after heat treatment at 80°C for 5 min.

Circular dichroism (CD) measurements

The CD spectra were recorded on a Jasco (Japan) J715 spectropolarimeter equipped with a temperature control system in continuous mode as described previously (Kim *et al*., 2000b; Park *et al*., 2002a). Far-UV CD measurements were carried out over the wavelength range 190–250 nm with 0.5 nm bandwidth, a 1 s response time and a 10 nm/min scan speed at 25 and 95°C. The spectra shown are an average of five scans that were corrected by subtraction of the buffer signal. Thermal denaturation experiments were performed using a heating rate of 1°C/min and a response time of 1 s. The CD spectra were measured every 0.5°C at a wavelength of 222 nm.

GST activity assay

The enzymatic activity of GST was assayed using a chromogenic substrate, 1-chloro-2,4-dinitrobenzene (CDNB), as described previously (Habig *et al*., 1974; Park *et al*., 2002a).

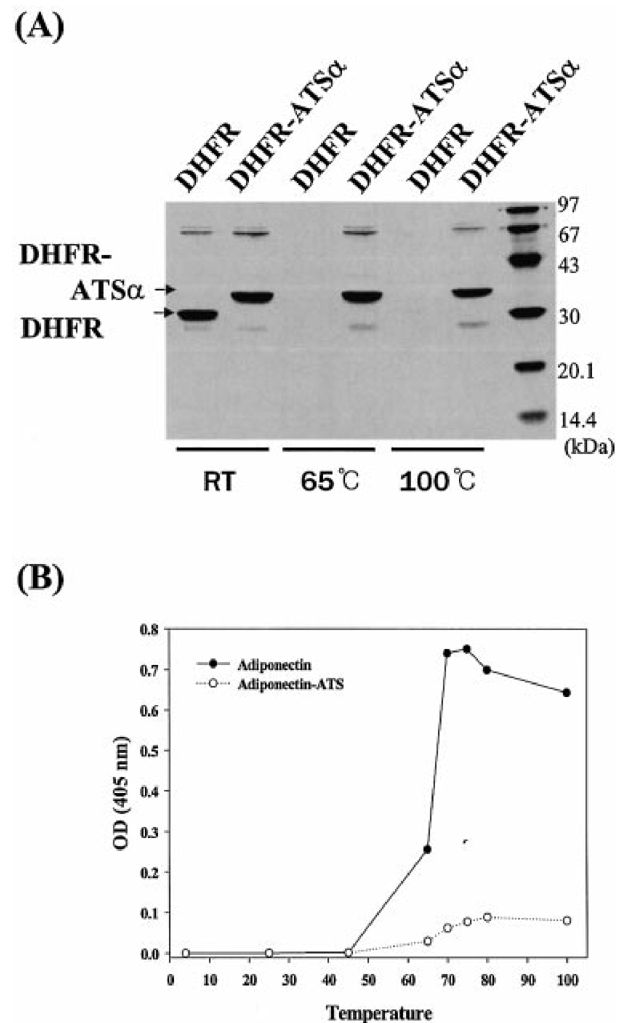

Fig. 1. Thermal behaviors of DHFR–ATSα and adiponectin–ATSα fusion proteins. (**A**) Heat resistance of the DHFR–ATSα fusion protein. The DHFR and DHFR–ATSα fusion proteins (0.6 mg/ml of each protein in PBS) were incubated for 10 min at room temperature (RT), 65°C and 100°C, respectively. The protein solutions were centrifuged and the supernatants were analyzed on a 12% SDS–polyacrylamide gel. The protein bands were stained with Coomassie Brilliant Blue R-250. (**B**) Heat resistance of the adiponectin–ATSα fusion protein. The adiponectin and adiponectin–ATSα fusion proteins (1.0 mg/ml of each protein in PBS) were incubated for 10 min at various temperatures and monitored on a spectrophotometer.

Results

Heat resistance of DHFR–ATSα and adiponectin–ATSα fusion proteins

Introducing the acidic tail of α-synuclein (ATSα, residues 96–140; also called Syn96–140) into GST appeared to protect the fusion protein from environmental stresses, such as heat, pH and metal ions (Park *et al*., 2002a). To investigate whether ATSα can be utilized to suppress the aggregation of other proteins, we first produced a DHFR–ATSα fusion protein and compared the thermal behaviors of DHFR and the DHFR–ATSα fusion protein using a qualitative heat-induced protein aggregation assay. Each protein was heat-treated in a water bath at 65 or 100°C for 10 min and the protein solution was centrifuged to remove the precipitates. Subsequently, the supernatant was analyzed on an SDS–polyacrylamide gel

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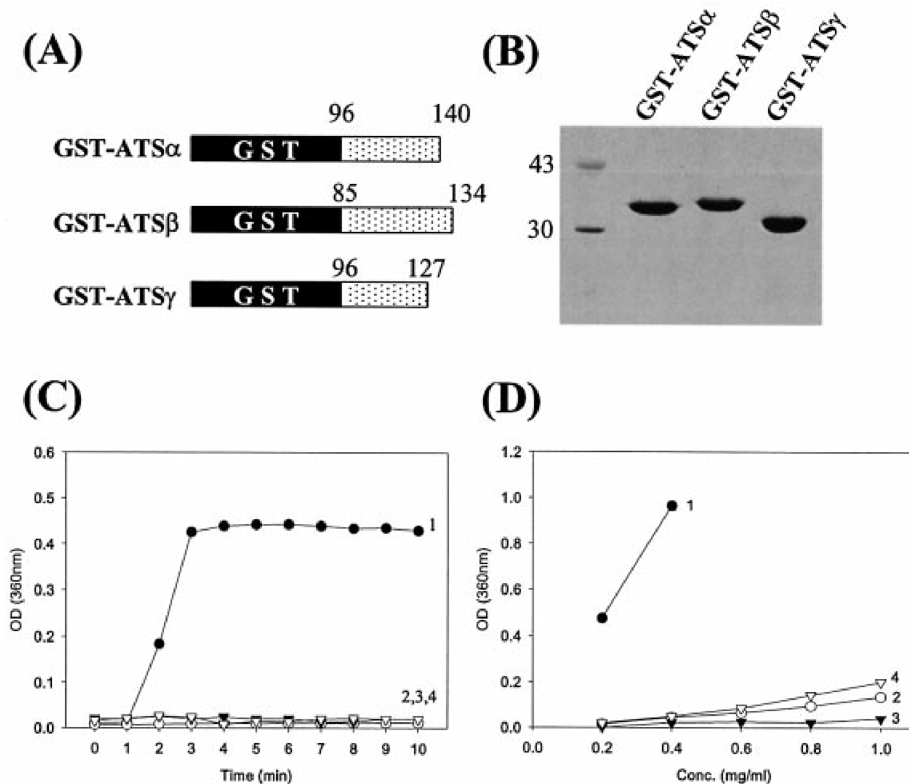

Fig. 2. Thermal behavior of GST–ATS fusion proteins. (**A**) Schematic diagram of GST–ATS fusion proteins. Starting and terminating residue numbers of ATS peptides are labeled. (**B**) SDS–PAGE analysis of GST–ATS fusion proteins after boiling for 10 min. (**C**) Heat-induced aggregation of the GST–ATS fusion proteins (0.2 mg/ml of each protein in PBS) was quantitatively assessed by monitoring the light scattering (OD_{360}) as a function of time at 65°C. (**D**) Heat-induced aggregation of GST–ATS fusion proteins was quantitatively assessed by monitoring the light scattering as a function of protein concentration after heating for 5 min at 80°C. (C, D) graphs: 1, GST; 2, GST–ATSα; 3, GST–ATSβ; and 4, GST–ATSγ.

(Figure 1A). As expected, DHFR–ATSα did not precipitate on heat treatment up to 100°C, whereas the DHFR protein completely precipitated at 65°C. We next produced a recombinant adiponectin and adiponectin–ATSα fusion protein and compared the heat resistance by monitoring the absorbance at 405 nm as a function of incubation temperature, while setting the concentration of each protein sample at 1.0 mg/ml. As shown in Figure 1B, the introduction of ATSα into the adiponectin appeared to protect the fusion protein significantly from heat-induced aggregation, when it was detected on a spectrophotometer. These results indicate that ATSα is a novel peptide conferring heat resistance on the fusion proteins.

Heat resistance of the GST–synuclein fusion protein containing the C-terminal acidic tail of β-synuclein or γ-synuclein

In addition to α-synuclein, β- and γ-synucleins and synoretin, which belong to the synuclein family, have also been identified in humans (Ueda *et al*., 1993; Jakes *et al*., 1994; Ji *et al*., 1997; Surguchov *et al*., 1999). The N-terminal amphipathic regions of the synuclein family members are well conserved among the species, but the C-terminal acidic tails are very diverse in size and in sequence (Lavedan, 1998; Hashimoto and Masliah, 1999; Iwai, 2000; Lücking and Brice, 2000). We next investigated whether GST–ATSβ and GST–ATSγ fusion proteins containing the acidic tail of β-synuclein (ATSβ, residues 85–134) and that of γ-synuclein (ATSγ, residues 96–127), respectively, are resistant to heat-induced aggregation (Figure 2A). GST–ATSβ and GST–ATSγ fusion proteins were qualitatively examined for heat resistance by SDS–PAGE, as described previously (Park *et al*., 2002a). As shown in Figure 2B, GST–ATSβ and GST–ATSγ as well as GST–ATSα do not precipitate at all after heat treatment at 100°C for 10 min, which indicates that they are extremely heat resistant. Subsequently, the thermal behaviors of the GST–ATS fusion proteins were quantitatively compared by monitoring their absorbance at 360 nm over time, while setting the concentration of each protein at 0.2 mg/ml at 65°C (Horwitz, 1992; Lee and Vierling, 1998; Kim *et al*., 2000a; Uversky *et al*., 2001; Park *et al*., 2002a). In this experiment, as shown in Figure 2C, the GST protein had almost aggregated after 2–3 min. In contrast, the GST–ATS fusion proteins did not aggregate at all even 10 min after heat treatment. Next, the GST–ATS fusion proteins were quantitatively assayed by monitoring the absorbance at 360 nm while varying the concentration from 0.2 to 1.0 mg/ml after heat treatment at 80°C for 5 min. As shown in Figure 2D, the GST–ATS fusion proteins did not precipitate at all after heat treatment, independently of the concentration, whereas the GST protein was completely precipitated at a low concentration. These results indicate that in addition to ATSα, the ATSβ and ATSγ peptides are also capable of providing heat resistance to other proteins and they can be used in the preparation of fusion proteins having resistance to environmental stresses. Since the amino acid sequence of synoretin is very similar to that of γ-synuclein

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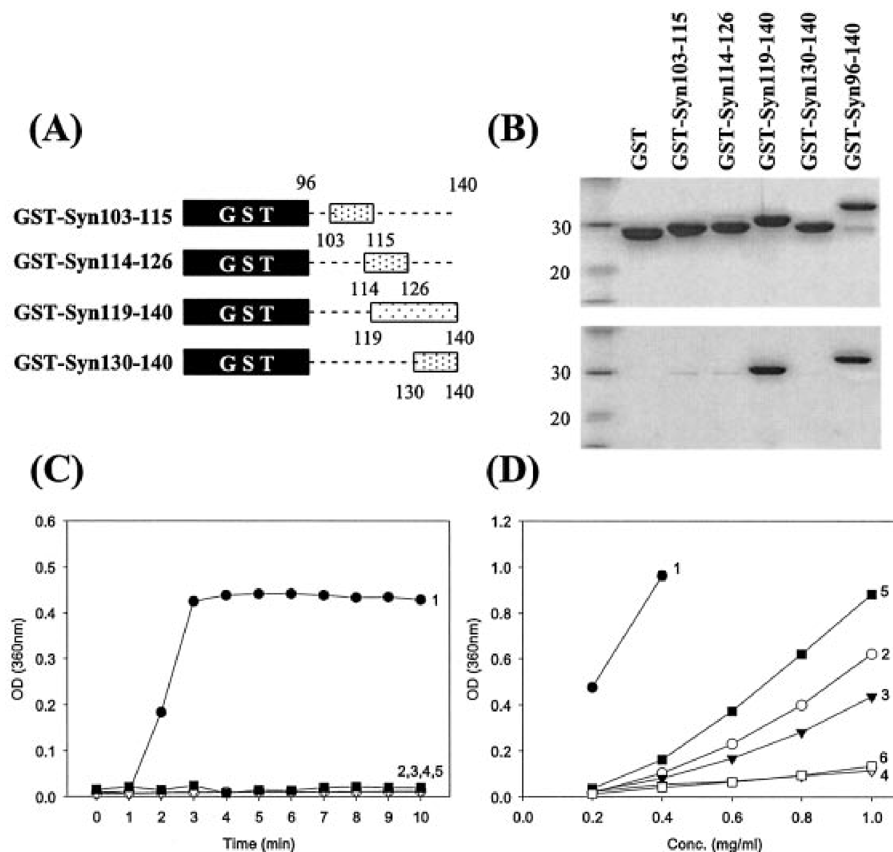

Fig. 3. Thermal behavior of GST–ATSα deletion mutants. (**A**) Schematic diagram of GST–ATSα deletion mutants. See text for details. (**B**) SDS–PAGE analysis of GST–ATSα deletion mutants before (above) and after (below) boiling for 10 min. (**C**) Heat-induced aggregation of the GST–ATSα deletion mutants (0.2 mg/ml of each protein in PBS) was quantitatively assessed by monitoring the light scattering (OD_{360}) as a function of time at 65°C. (**D**) Heat-induced aggregation of the GST–ATSα deletion mutants was quantitatively assessed by monitoring the light scattering as a function of protein concentration after heating for 5 min at 80°C. (C, D) graphs: 1, GST; 2, GST–Syn103–115; 3, GST–Syn114–126; 4, GST–Syn119–140; 5, GST–Syn130–140; and 6, GST–Syn96–140.

(Surguchov *et al*., 1999), the acidic tail of synoretin may have a similar property.

Heat-resistance of GST–synuclein fusion proteins with shorter peptide fragments derived from the ATSα

The C-terminal acidic tail of α-synuclein (ATSα) is composed of 45 amino acids (residues 96–140) and 15 Glu/Asp residues are scattered throughout the ATSα (Ueda *et al*., 1993; Jakes *et al*., 1994). We next investigated the thermal behaviors of deletion mutants of the GST–ATSα fusion protein which have shorter ATSα peptide fragments. For this purpose, a series of GST–ATSα deletion mutants were produced using peptide fragments which span the highly charged regions of ATSα (Figure 3A). GST–Syn103–115 contains five Glu/Asp residues out of a total of 13 amino acids contained in a fragment of ATSα (residues 103–115); GST–Syn114–126 contains 6 Glu/Asp residues out of a total of 13 amino acids contained in a fragment of ATSα (residues 114–126); GST–Syn119–140 contains nine Glu/Asp residues out of a total of 22 amino acids contained in a fragment of ATSα (residues 119–140); and GST–Syn130–140 contains five Glu/Asp residues out of a total of 11 amino acids contained in a fragment of ATSα (residues 130–140). Isoelectric points (p*I*) of these fusion proteins are shown in Table II. When these GST–ATSα deletion mutants were thermally treated at a high concentration (0.8 mg/ml), GST–Syn96–140, which contains the entire region of ATSα, and GST–Syn119–140, which contains 22 amino acids of ATSα, did not precipitate at all, whereas GST–Syn103–115, GST–Syn114–126 and GST–Syn130–140, which contain 11–13 amino acids, precipitated almost completely (Figure 3B). On the other hand, when these GST–ATSα deletion mutants

Table II. Isoelectric point (p*I*) and the temperature for the onset of unfolding (T_u) values of GST, GST–ATSα deletion mutants and GST–polyE fusion proteins

Protein	p*I* value	T_u value[a] (°C)	Heat resistance
GST	6.18	54[b]	–
GST–Syn103–115	5.35	54	++
GST–Syn114–126	5.26	58	++
GST–Syn119–140	5.03	58	++++
GST–Syn130–140	5.35	55	++
GST–E5	5.38	57	+
GST–E10	5.02	56	++
GST–ATSα	4.85	54[b]	++++
GST–ATSβ	4.79	ND[c]	++++
GST–ATSγ	5.52	ND	++++

[a]T_u values were obtained by linear extrapolation of the melting curves (Figure 6) to the temperature axis.
[b]Determined from previously reported data (Park *et al*., 2002a).
[c]ND, not determined.

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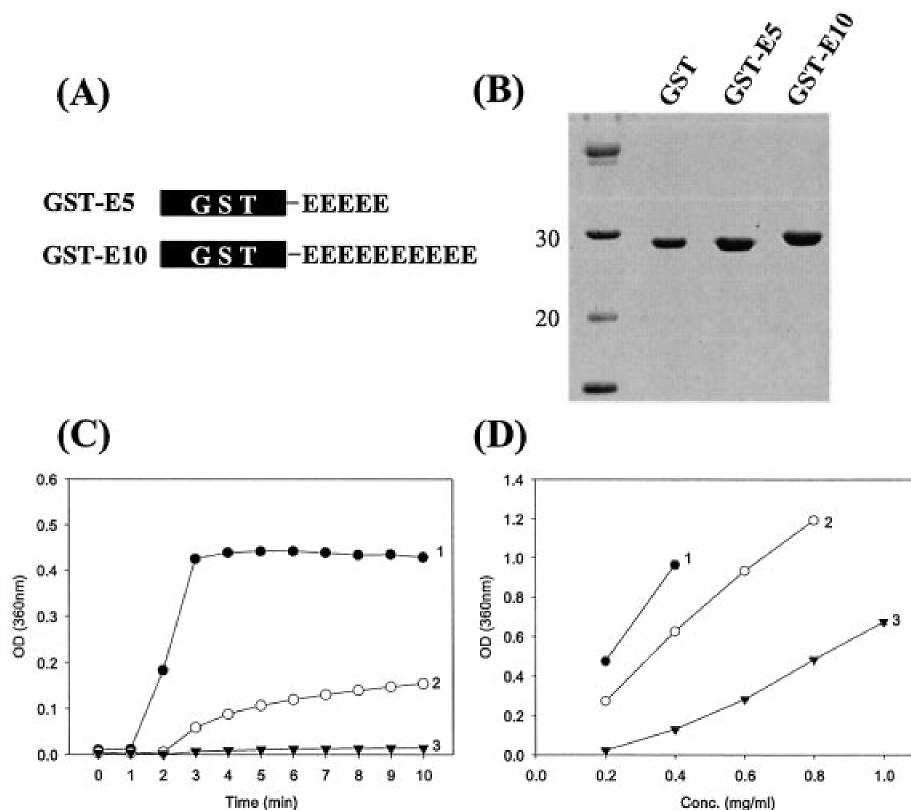

Fig. 4. Thermal behavior of GST–polyE fusion proteins. (**A**) Schematic diagram of GST–poly E (E5, E10) fusion proteins. (**B**) SDS–PAGE of GST, GST–E5 and GST–E10. (**C**) Heat-induced aggregation of GST, GST–E5 and GST–E10 (0.2 mg/ml of each protein in PBS) was quantitatively assessed by monitoring the light scattering (OD_{360}) as a function of time at 65°C. (**D**) Heat-induced aggregation of GST, GST–E5 and GST–E10 was quantitatively assessed by monitoring the light scattering as a function of protein concentration after heating for 5 min at 80°C. (C, D) graphs: 1, GST; 2, GST–E5; and 3, GST–E10.

were thermally treated at a low concentration (0.2 mg/ml), none of the fusion proteins aggregated (data not shown).

The thermal behaviors of the GST–ATSα deletion mutants were quantitatively analyzed by monitoring the absorbance at 360 nm over time, while setting the concentration of each protein at 0.2 mg/ml at 65°C. As shown in Figure 3C, the OD_{360} of the GST protein drastically increased 2 min after heat treatment and most of the protein had aggregated by 3 min. In contrast, the GST–ATSα deletion mutants did not aggregate at all even 10 min after heat treatment. Next, the GST–ATSα deletion mutants were quantitatively assayed by monitoring the absorbance at 360 nm, while varying the concentration from 0.2 to 1.0 mg/ml after heat treatment at 80°C for 5 min. As shown in Figure 3D, GST–Syn96–140, which contains the entire region of ATSα, and GST–Syn119–140, which contains 22 amino acids of ATSα, did not precipitate at all after heat treatment under these conditions, whereas GST–Syn103–115, GST–Syn114–126 and GST–Syn130–140, which contain 11–13 amino acids of ATSα, did not precipitate at a low concentration, but increasingly aggregated as the concentration rose. It is well known that the extent of protein aggregation is proportional to the concentration (Zettlmeissl *et al*., 1979; Kiefhaber *et al*., 1991). Hence it was demonstrated that the GST–ATSα deletion mutants have heat resistance superior to that of wild-type GST and, interestingly, that the heat resistance varies depending on the length and charge density of ATSα. Therefore, optimum effects can be achieved by suitably selecting the length and charge density of ATSα according to the size and hydropathy of the target protein.

Thermal behaviors of GST–polyglutamate fusion proteins

As demonstrated in Figure 3, the heat resistance of the GST–ATSα deletion mutants appeared to be approximately proportional to the number of Glu/Asp residues in the ATSα fragments. To address the importance of negative charge in the process of conferring heat resistance on the fusion proteins, we next examined whether GST fusion proteins with genuinely negatively charged peptide fragments such as polyglutamate are heat resistant. For this, a series of GST–polyglutamate fusion proteins were constructed by ligating the gene part of polyglutamate into pGEX vector (Figure 4A). Purified proteins of GST–E5 (containing five consecutive glutamate residues) and GST–E10 (containing 10 consecutive glutamate residues) are demonstrated in Figure 4B and the p*I* values of these fusion proteins are shown in Table II. Each protein suspended in PBS (0.8 mg/ml) was heated in a boiling water-bath for 10 min and then cooled in air. The protein samples were centrifuged at 15 000 r.p.m. for 10 min and the supernatants were analyzed on a 12% SDS–polyacrylamide gel. Neither GST–E5 nor GST–E10 showed any protein bands after heat treatment, which indicates that they had been completely precipitated by heat treatment (data not shown). This indicates that, unlike the GST–ATSα fusion proteins, neither GST–E5 nor GST–E10 is heat resistant under such stringent conditions.

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The thermal behaviors of the GST–E5 and GST–E10 fusion proteins were quantitatively assayed by monitoring their absorbance at 360 nm over time, while setting the concentration of each protein at 0.2 mg/ml at 65°C. As shown in Figure 4C, the GST protein was completely aggregated after 2–3 min and the GST–E5 fusion protein was aggregated to a considerable extent under the same conditions, whereas the GST–E10 fusion protein did not aggregate at all under these conditions. Next, the GST–polyglutamate fusion proteins were quantitatively assayed by monitoring their absorbance at 360 nm, while varying the concentration from 0.2 to 1.0 mg/ml after heat treatment at 80°C for 5 min. As shown in Figure 4D, the GST protein was completely precipitated at a low concentration and most of the GST–E5 protein was precipitated at a high concentration. In contrast, the GST–E10 protein was partially precipitated after heat treatment under these conditions and increasingly aggregated as the concentration rose. Hence as the length of the polyglutamate increases, the negative charge considerably increases, thus causing the amount of aggregation decrease. Interestingly, however, it is noted that the polyglutamate tail is considerably less effective in providing heat resistance, as compared with ATS peptides containing the same number of glutamate residues (Figures 3 and 4; Table II). In fact, GST–Syn130–140 shows heat resistance far superior to that of GST–E5, which contains the same number of glutamate residues, and slightly better heat resistance than that of GST–E10, which contains twice as many glutamate residues (compare Figure 3D with Figure 4D). Therefore, it is suggested that the characteristic amino acid sequence of ATS, in addition to the increased solubility of the proteins due to the increase in the negative charge, plays an important role in conferring resistance to heat-induced aggregation.

CD spectra of GST–Syn119–140 and GST–E10

To address the conformational properties of the introduced peptide tags, we compared the CD spectra of representing fusion proteins (Figure 5). The far-UV CD spectra of GST–Syn119–140 indicate that the protein contains well-ordered secondary structure elements (absorption bands at 210–220 nm in Figure 5A). The CD spectrum at room temperature appeared to be very similar to that of GST (figure 7A in Park *et al.*, 2002a). Interestingly, however, the far-UV CD spectra of the GST–E10 exhibit an additional absorption band at 195 nm, which is characteristic of random-coiled polypeptides (Figure 5B). These results suggest that the Syn119–140 peptide in GST–Syn119–140 may be packed together with the GST domain, but the E10 peptide in GST–E10 may be extended from the GST domain to the solvent forming a random-coil peptide.

Secondary structural changes of GST–ATSα deletion mutants and GST–polyE fusion proteins

The secondary structural changes of the GST–ATSα deletion mutants and GST–polyE fusion proteins induced by the increase of temperature were investigated by CD spectroscopy. Specifically, to compare the thermal stabilities of the fusion proteins, the thermal unfolding of each protein was monitored at 222 nm as a function of temperature (Figure 6). Consistent with a previous report (figure 7A in Park *et al.*, 2002a), the temperature-induced unfolding of GST started around 54°C and the CD signal kept diminishing until 100°C due to the complete precipitation of the protein (data not shown). Unlike in the case of wild-type GST protein, however, the temperature-induced unfolding of the GST–ATSα deletion mutants all took place in two stages (Figure 6A–D). The first transition started at ~54–58°C, whereas the second transition occurred at >90°C. The melting curves of GST–polyE fusion proteins appeared to be similar to those of the GST–ATSα deletion mutants (Figure 6E and F), although the secondary transition was less clearly seen. It is highly likely that the secondary transition of GST is not able to be observed, since the protein precipitates before that temperature. Interestingly, the first transition temperatures of the GST–ATSα deletion mutants and GST–polyE fusion proteins all appeared to be similar or slightly higher than that of wild-type GST, suggesting that the introduced acidic tails do not significantly affect the intrinsic stability of the protein.

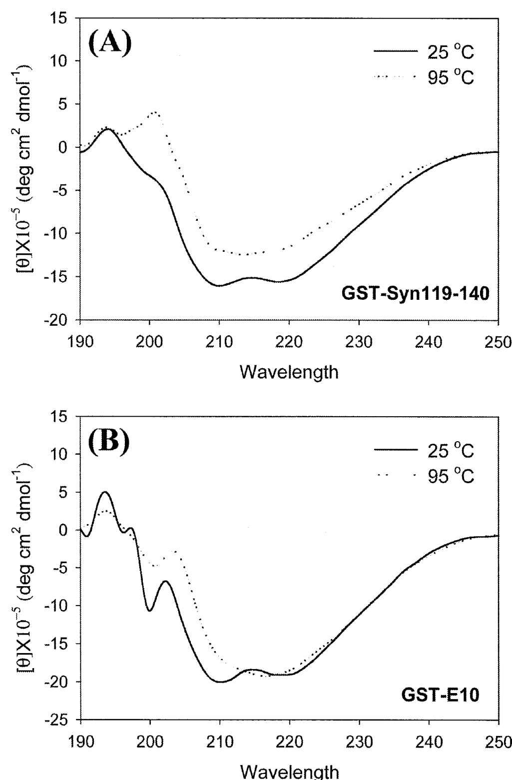

Fig. 5. Far-UV CD spectra of (**A**) GST–Syn119–140 and (**B**) GST–E10. Far UV-CD spectra measured at 25 and 95°C are drawn as solid and dotted lines, respectively.

Enzyme activity of GST–ATSα deletion mutants after heat-treatment

We next compared the thermostability of the GST–ATSα deletion mutants by measuring their thermal inactivation curves (Figure 7), which were used to determine the T_{50} values, i.e. the temperatures at which 50% of the initial enzymatic activity is lost after heat treatment. As shown in Figure 7, the T_{50} values of the GST–ATSα deletion mutants are only slightly higher than that of wild-type GST. This suggests that the stabilizing effect of ATSα peptides on the enzymatic activity is not particularly high, at least in the case of GST.

Discussion

Previous studies have demonstrated that the C-terminal acidic tail of α-synuclein (ATSα) is capable of protecting the GST–ATSα fusion protein from stress-induced aggregation (Park

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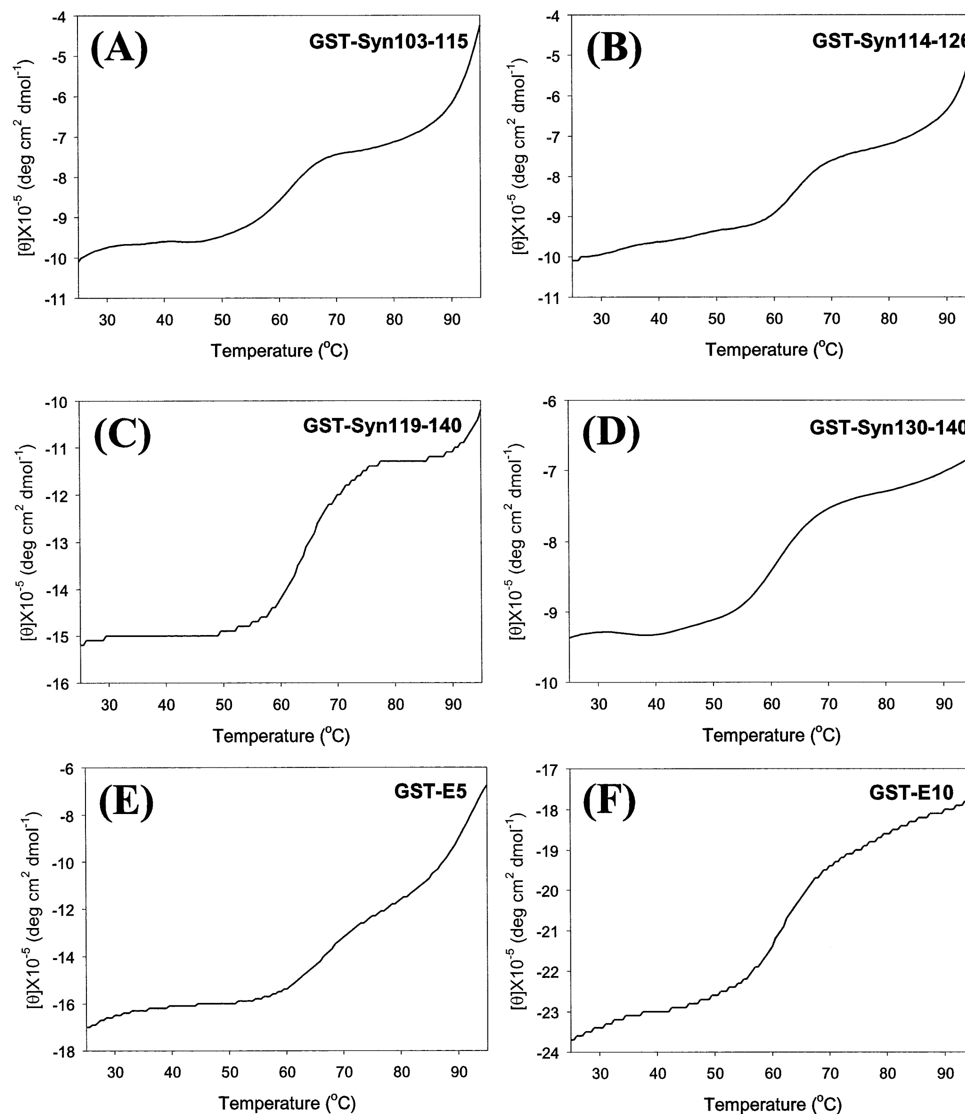

Fig. 6. Melting curves of GST–ATSα deletion mutants and GST–polyE fusion proteins. The mean molar ellipticity per residue of each protein (0.4 mg/ml in PBS) at 222 nm was measured as a function of temperature. The solid lines represent temperature scans from 20 to 100°C (heating mode). (**A**) GST–Syn103–115; (**B**) GST–Syn114–126; (**C**) GST–Syn119–140; (**D**) GST–Syn130–140; (**E**) GST–E5; and (**F**) GST–E10.

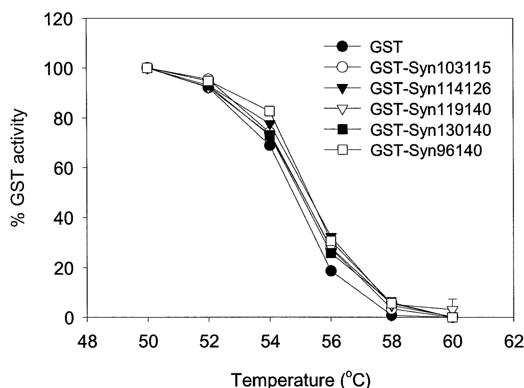

Fig. 7. Thermal inactivation of the GST–ATSα deletion mutants. Activity is expressed as a percentage of initial activity. Values are the means of three independent experiments with the standard deviation shown as bars. The protein samples were incubated for 5 min at indicated temperatures before enzyme assay.

et al., 2002a). In this study, we have shown that the introduction of ATSα into other proteins, such as DHFR and adiponectin, renders the fusion proteins resistant to heat-induced aggregation. In addition to ATSα, ATSβ and ATSγ also appear to protect the fusion protein from heat-induced aggregation. These ATS fusion proteins also appear to be resistant to pH- and metal-induced aggregation (our unpublished results). These results indicate that ATS peptides are novel peptides, conferring resistance to environmental stresses, such as heat, pH and metal ions. These results also imply that many biologically or medically important proteins, particularly those having aggregation problems, could be transformed into a more resistant protein by introducing the ATS peptides.

The C-terminal acidic tails of the synuclein family members are diverse in size and sequence, but they are all highly charged with many Glu/Asp residues (Lavedan, 1998; Hashimoto and Masliah, 1999; Iwai, 2000; Lücking and Brice, 2000). Earlier studies have shown that protein solubility is approximately proportional to the square of the net charge on the protein

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(Tanford, 1961). Therefore, the abolishment of heat-induced aggregation in ATS containing fusion proteins at high temperatures seems to be primarily due to the presence of the negatively charged residues in the ATS peptides, since these negative charges increase the solubility of the protein, by increasing its hydrophilicity and by causing intermolecular interactions to be unfavorable. This possibility is supported by the fact that the GST–ATSα deletion mutants, which contain shorter peptides derived from the highly charged regions of ATSα, all appear to be relatively heat resistant. In particular, the heat resistance of the GST–ATSα deletion mutants is approximately proportional to the number of Glu/Asp residues in the ATSα fragments in addition to the peptide chain length. For example, GST–Syn119–140, which contains nine Glu/Asp residues out of a total of 22 amino acids contained in a fragment of ATSα, is extremely heat resistant, as in the case of GST–Syn96–140. In contrast, GST–Syn103–115, GST–Syn114–126 and GST–Syn130–140, which contain 5–6 Glu/Asp residues out of a total of 11–13 amino acids contained in a fragment of ATSα, are less heat resistant. Consequently, the heat resistance of the GST–ATSα deletion mutants is correlated with their p*I* values (Table II). These results indicate that the negative charges contributed by the Glu/Asp residues in the ATSα-derived peptides are crucial for conferring heat resistance on the fusion proteins.

However, the negative charges in the ATSα-derived peptides appear insufficient to explain the extreme heat resistance of the fusion proteins. As shown in Figure 4, GST–E5, which contains five consecutive Glu residues, is much less heat resistant than GST–Syn130–140 or GST–Syn103–115, which contain the same number of Glu/Asp residues. Similarly, GST–E10, which contains 10 consecutive Glu residues, is much less heat resistant than GST–Syn119–140, which contains nine Glu/Asp residues. The heat resistance of GST–E10 is fairly comparable to that of GST–Syn130–140. These results suggest that not only the charged residues, but also the specific amino acid sequence of ATSα, plays an important role in conferring extreme heat resistance on the fusion proteins. It would be interesting to investigate in more detail just why the ATS peptides fused in GST are superior to other highly charged peptides for protecting the fusion proteins from stress-induced aggregation.

To address the above question, we compared the far-UV CD spectra of GST–Syn119–140 and GST–E10 and found that the conformations of the introduced peptide tags might be different (Figure 5). The CD spectra of GST–Syn119–140 and GST–E10 suggest that the Syn119–140 peptide might be packed with the GST domain in the fusion protein, but the E10 peptide might protrude from the GST domain, forming an exposed random coil-like conformation. This reflects that, unlike the E10 peptide, Syn119–140 peptide has a potential to interact with other proteins, as has been implied by previous studies (reviewed in Lücking and Brice, 2000). Presumably, the hydrophobic residues, which are scattered throughout the ATS, play an important role in the ATS peptide–protein interactions. Based on this observation, it is tempting to speculate that the intramolecular and/or intermolecular peptide–protein interactions mediated by the characteristic amino acid sequence of ATS peptides play an additional role in conferring the extreme heat resistance on the fusion proteins.

The effects of ATSα peptides on the stability of the fusion proteins were assessed by analyzing their heat-induced secondary structural changes and thermal inactivation curves (Figures 6 and 7, respectively). To compare the stabilities of the proteins, it is useful to determine the melting temperature (T_m) of each protein by CD spectroscopy or calorimetric analysis. T_m has been widely used as a thermodynamic parameter of the conformational stability of the protein. However, T_m can be correctly determined only for the reversible transition. For the irreversible transition, T_m is meaningless and often contains numerous errors. For example, the heat-induced unfolding of GST starts at around 54°C and the CD signal at 222 nm keeps diminishing until 100°C as the protein precipitates (Park *et al*., 2002a). The T_m value determined from this melting curve cannot but be overestimated. To describe quantitatively the protein stability for the irreversible transition, we therefore compared the derived temperatures for the onset of unfolding (T_u values), which were obtained by linear extrapolation of the melting curves to the temperature axis (Table II), as previously tried by Chrunyk and Wetzel to determine the derived temperatures for the onset of aggregation (Chrunyk and Wetzel, 1993). The T_u values of the GST–ATSα deletion mutants and GST–polyE fusion proteins appear to be around 54–58°C (Figure 6; Table II), whereas that of GST is about 54°C (figure 7A in Park *et al*., 2002a). These results indicate that the introduction of the acidic tails does not significantly affect the intrinsic stability of the protein. Rather, they appear to somewhat stabilize the fusion proteins. Analysis of the thermal inactivation curves results in a similar conclusion (Figure 7). The T_{50} values, the temperature at which 50% of the initial enzyme activity is lost after heat treatment, of the GST–ATSα deletion mutants appear to be very similar to or slightly higher than that of wild-type GST. These results also indicate that the introduction of ATSα-derived peptides does not significantly affect the intrinsic stability of the protein.

In summary, we have demonstrated that the introduction of the ATS peptides into heat-labile proteins protects the fusion proteins from heat-induced aggregation. Furthermore, our data suggest that the ATS peptides do not significantly affect the intrinsic stability of the fusion proteins. Introducing the ATS peptides will also contribute to the protein's solubility, since it greatly increases the hydrophilicity of the protein and makes intermolecular interactions unfavorable through electrostatic repulsion. Therefore, the ATS peptides 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 ATS peptides. Introducing the ATS peptides could make the protein more robust and enhance its shelf-life and duration time *in vivo*. Consequently, introducing the ATS peptides could also make the protein more amenable to use in alternative delivery methods and formulations.

Acknowledgements

We thank Dr R.Jakes (MRC, Cambridge) for the recombinant DNA of α-synuclein and Dr H.S.Rhim (Catholic University, Seoul, Korea) for the GST–Syn96–140 construct. This work was supported in part by a grant from KOSEF (R01-1999-000-00129-0) and by a grant from ATGen Co. Ltd.

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Received December 16, 2003; revised February 27, 2004; accepted March 16, 2004

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