Thermal Behavior of Proteins: Heat-Resistant Proteins and Their Heat-Induced Secondary Structural Changes†

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ABSTRACT: Most proteins are denatured by heat treatment, and the process is usually irreversible. However, some proteins, such as hyperthermophilic proteins are known to be stable even at the boiling temperature of water. We here describe a systematic investigation of thermal behavior of proteins by purifying and characterizing some heat-resistant proteins (HRPs) that are not aggregated upon heat treatment. Although most proteins were precipitated by boiling in a water bath, about 20 and 70 wt % of total proteins appeared to be heat-resistant in Jurkat T-cell lysates and human serum, respectively. We identified major HRPs from Jurkat T-cells and human serum by N-terminal amino acid sequencing and Western blot analysis. HRPs of 20 and 45 kDa (HRP20 and HRP45) were identified as α-synuclein and calreticulin, respectively, and HRPs of 60, 27, and 16 kDa (HRP60, HRP27, and HRP16) were identified as human serum fetuin, apolipoprotein A-I, and transthyretin, respectively. By a systematic investigation of the effect of heat on the secondary structure of the purified HRPs by circular dichroic spectroscopy, we observed four major types of thermal behavior, suggesting that the proteins could protect themselves through these pathways. Although our analysis is restricted to protein secondary structural changes, our data indicate that heat resistance of protein can be achieved in several different ways depending on the thermodynamic stability of native (N), unfolded (U), denatured (D), and intermediate (I) states.

Most proteins are denatured by boiling in an aqueous solution, and the process is usually irreversible (*1*). The heat-induced protein denaturation is easily experienced in everyday lives by insoluble protein aggregates, such as fried eggs and tofu. At high temperature, unfolding of the protein occurs that often results in insoluble aggregation or another state of tertiary structure through various folding pathways depending on the nature of the unfolded state. Although most proteins are heat-labile, some proteins are known to be stable even at the boiling temperature of water. For example, proteins from many hyperthermophilic organisms are heat-stable with extremely high T_m values relative to their mesophilic counterparts (*2, 3*). Most enzymes from these organisms are active for many hours at or above 100 °C, suggesting that they must have special mechanisms for heat stability (*4, 5*). Thorough study of these proteins might therefore reveal the structural determinants of heat stability at very high temperatures.

Previous studies have shown that the way proteins achieve heat stability seems to be different from protein to protein (*6*−*8*). Several attributes frequently proposed to explain the heat stability of proteins include relatively small solvent-exposed surface area, increased packing density that reduces cavities in the hydrophobic core, an increase of hydrophobicity in the core region, decreased length of surface loops, and increased hydrogen bonds between polar residues. More specifically, amino acids at nonburied or surface positions in α-helices have been implicated to play a dominant role in obtaining heat stability (*9*−*11*). Appropriate substitution of those amino acids turned out to make the α-helices become more rigid and tightly packed with increased hydrophobic contacts. Thus, those α-helices would provide the earliest nucleating centers critical for folding and later act as an exposed rigid framework surrounding and interacting with the protein core, and they would keep the protein compact and resistant to denaturation at higher temperature, as has been suggested by Viguera et al. (*12*). In general, the secondary structural elements such as α-helices, β-sheets, and ω-loops have been known to act as cooperative folding units (*13*−*17*). Tertiary interactions also greatly stabilize secondary structural elements, and their cooperative nature is intrinsic to the structure and proper folding of proteins (*18*). Therefore, monitoring the change of secondary structure during the heating process will provide information that could be exploited to understand the relationship of protein structure and heat stability.

In this paper, we systematically investigated the thermal behavior of proteins by purifying and characterizing some heat-resistant proteins (HRPs)[1] from Jurkat T-cells and human serum that are not aggregated by heat treatment. Surprisingly, many proteins appeared to be heat-resistant in

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[1] Abbreviations: HRP, heat-resistant protein; CD, circular dichroism; HSF, human serum fetuin; apoA-I, apolipoprotein A-I; BSA, bovine serum albumin; HSA, human serum albumin; BSF, bovine serum fetuin; GuHCl, guanidine hydrochloride; T_m, melting temperature.

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both Jurkat cell lysates and human serum. A systematic investigation of the effect of heat on the purified HRPs enabled us to observe four major types of thermal behavior of HRPs, indicating that heat resistance of protein can be achieved in several different ways. Group I is represented by the proteins having a high proportion of random coil, such as α-synuclein and α_s-casein. The conformation of group II is irreversibly changed by heat treatment, and group III is characterized by a reversible conformational change upon heat treatment. Group IV is characterized by the absence of conformational change upon heat treatment, since the proteins are quite stable even at the boiling temperature of water. The heat-induced states of all group I−IVs are stable and soluble at high temperature, while those of heat-labile proteins are so unstable that they result in precipitation or aggregation.

MATERIALS AND METHODS

Materials. The human T-cell lymphoma cell line Jurkat was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in culture with RPMI 1640 medium (Flow Laboratories, Rockville, MD) supplemented with 10% (v/v) FBS, 1% L-glutamine, and 1% antibiotic solution. Glutamate racemase, a hyperthermophilic protein from *Aquifex pyrophilus* (*19*), was a generous gift from Dr. Y. J. Cho (KIST, Seoul, Korea). α_s-Casein and BSA were obtained from Sigma (St. Louis, MO).

Identification of Heat-Resistant Proteins from Jurkat T-cells. Jurkat cells (1×10^8 cells) were lysed in 1 mL of lysis buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM iodoacetamide, 1 mM PMSF, 5 μg/mL leupeptin, and 5 μg/mL pepstatin. Lysates were centrifuged at 14000*g* for 20 min, and the supernatants were heated in a boiling water bath for 10 min. Precipitates were removed by centrifugation at 12 000 rpm for 20 min, and the supernatant was applied to a DEAE-Sepharose CL6B column preequilibrated in 20 mM Tris-HCl buffer (pH 7.5) with 0.1 M NaCl. The column was washed with the same buffer and the fractions containing unbound proteins were collected (peak I). Bound proteins were sequentially eluted with 0.2 M NaCl (peak II), 0.3 M NaCl (peak III), and 0.5 M NaCl (peak IV) in the same buffer. Fractions containing major HRPs were identified by Coomassie blue staining of SDS−polyacrylamide gels (Figure 1B). The partially purified major HRPs were subjected to N-terminal amino acid sequence analysis.

Identification and Purification of α-Synuclein. Partially purified Jurkat lysates (peak I and II fractions) were separated on an SDS−12% polyacrylamide gel, and proteins were transferred onto poly(vinylidene difluoride) (PVDF) membranes (Immobilon-P; Millipore, Marlborough, MA). Western blot analysis was performed with polyclonal anti-α-synuclein antibodies raised against the purified recombinant protein as previously described (*20*). The blots were developed with secondary horseradish peroxidase-conjugated sheep anti-rabbit IgG (Amersham, Buckinghamshire, England) and ECL substrate (Amersham) following the manufacturer's recommendation. α-Synuclein was overexpressed in *Escherichia coli* and the recombinant protein was purified as previously described (*21*, *22*).

Identification of Heat-Resistant Proteins from Human Serum. Human serum (50 mL) was diluted in 2 volumes of 20 mM Tris (pH 7.5) buffer with 0.1 M NaCl and heated in a boiling water bath for 10 min. Following a 20 min centrifugation at 12 000 rpm, the supernatant was applied to a DEAE-Sepharose CL6B column preequilibrated in 20 mM Tris-HCl buffer (pH 7.5) with 0.1 M NaCl. Bound proteins were sequentially eluted with 0.2 M NaCl (peak I) and 0.3 M NaCl (peak II) in the same buffer. Most proteins were eluted under these conditions. Peak I fractions containing the HRPs of 60, 40, and 27 kDa (HRP60, HRP40, and HRP27, respectively) were pooled, concentrated, and further purified on a Sephacryl S200 gel-filtration column. Peak II fractions containing the HRPs of 60 and 16 kDa (HRP60 and HRP16) were also pooled, concentrated, and further purified on a Sephacryl S200 gel-filtration column. Fractions containing each HRP were identified by Coomassie blue staining of SDS−polyacrylamide gels. The partially purified HRPs were subjected to N-terminal amino acid sequence analysis.

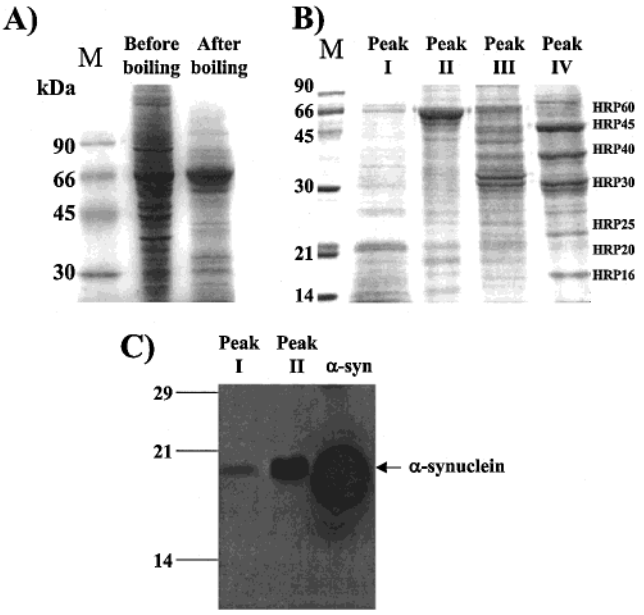

FIGURE 1: Heat-resistant proteins of Jurkat T-cell lysates. (A) SDS−PAGE analysis of Jurkat T-cell lysates before and after heat treatment. **(B)** Partial purification of HRPs from Jurkat cell lysates on an anion-exchange column. Fractions of peaks I−IV were pooled and analyzed on an SDS−polyacrylamide gel. **(**C) Identification of HRP20. Western blot analysis reveals that HRP20 is α-synuclein, an intrinsically unstructured protein.

N-Terminal Amino Acid Sequencing. HRPs were sequenced from the N-terminus by the automatic Edman degradation procedure on an ABI 476A peptide sequencer in the Korea Basic Science Center (Seoul, Korea). Briefly, partially purified HRPs were separated on SDS−polyacrylamide gels and transferred onto PVDF membranes. The protein bands were sliced and subjected to the automatic Edman degradation procedure.

Purification of Human Serum Fetuin. Human serum fetuin (HSF, also called α2-HS glycoprotein) was purified as described by Kellermann et al. (*23*) with slight modification. Briefly, 100 mL of human serum was diluted in two volumes of 20 mM Tris (pH 7.5) buffer, and the solution was applied to a DEAE-Sepharose anion-exchange column equilibrated in 20 mM Tris-HCl buffer (pH 7.5). Bound protein was eluted with a linear gradient of 0.1−0.4 M NaCl in 20 mM Tris (pH 7.5). Fractions of HSF identified by Coomassie blue

staining of SDS−polyacrylamide gels were pooled and concentrated on Centriprep 30 concentrators (Amicon, Beverly, MA). The concentrated HSF sample was further purified on a Superdex-200 FPLC gel-filtration column (Pharmacia) equilibrated in 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl. The HSF fractions were identified by Coomassie blue staining of SDS−polyacrylamide gels containing aliquots of each fraction. The HSF fractions were pooled and subjected to a Resource Q anion-exchange column. Bound protein was eluted with a linear gradient of 0.1−0.3 M NaCl in 20 mM Tris (pH 7.5), and the HSF fractions were pooled and concentrated on a Centriprep 30 concentrator.

Purification of Apolipoprotein A-I. ApoA-I was purified as described by Wetterau and Jonas (*24*) with slight modification. Briefly, high-density lipoprotein (HDL) fraction was prepared from 50 mL of human serum by ultracentrifugal flotation in KBr between densities 1.063 and 1.210 g/mL. From the HDL fraction, apoA-I was isolated on the Superdex-200 (Pharmacia) FPLC column equilibrated in 10 mM Tris-HCl buffer (pH 8.5) with 6 M GuHCl. The fractions were analyzed by SDS−PAGE, and the fractions containing the apoA-I were applied to a Q-Sepharose column equilibrated in 10 mM Tris-HCl buffer (pH 7.5) with 6 M GuHCl. The proteins were eluted with a linear gradient of 0−0.5 M NaCl in 10 mM Tris-HCl (pH 7.5) buffer with 6 M GuHCl. At this stage, most of the high molecular weight impurities were removed. The apoA-I fractions were pooled and dialyzed against 10 mM phosphate buffer (pH 7.5) for 12 h at 4 °C. After dialysis, the protein solution was concentrated and further purified on a Superdex-75 (Pharmacia) FPLC gel-filtration column equilibrated in 10 mM Tris-HCl buffer (pH 7.5).

Purification of Transthyretin. Transthyretin was purified from human serum by a combination of ammonium sulfate fractionation and conventional chromatography techniques (*25*). Human serum was fractionated by 40% ammonium sulfate treatment, and the supernatant was dialyzed against 20 mM Tris (pH 7.5) buffer containing 0.1 M NaCl at 4 °C for 24 h and then loaded onto a DEAE-Sephacryl CL6B column preequilibrated with the same buffer. Bound protein was eluted with a linear gradient of 0.1−0.4 M NaCl in 20 mM Tris (pH 7.5). Transthyretin fractions identified by Coomassie blue staining of SDS−polyacrylamide gels were pooled and concentrated on Centriprep 10 concentrators (Amicon). The concentrate was further purified on a Superdex-200 FPLC gel-filtration column (Pharmacia) equilibrated in 20 mM PBS (pH 7.4). Transthyretin fractions were pooled and subjected to a Resource Q anion-exchange column. Bound protein was eluted with a linear gradient of 0.1−0.4 M NaCl in 20 mM Tris (pH 7.5), and the transthyretin fractions were pooled and dialyzed against 20 mM PBS (pH 7.5).

CD Measurements. CD spectra were recorded on a Jasco-J715 spectropolarimeter (Jasco, Japan) equipped with a temperature control system in a continuous mode. Far-UV CD measurements were carried out over the wavelength range of 190 to 250 nm with 0.5 nm bandwidth, 1 s response time, and 10 nm/min scan speed at 20 °C unless otherwise specified. Spectra were the average of five scans that were corrected by subtraction of the buffer signal. 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 varied between 1 and 20 μM to monitor a potential aggregation effect. Thermal denaturation experiments were performed with a heating rate of 1 °C/min and a response time of 1 s. Thermal scan data were collected from 20 to 100 °C in 2 mm path-length cuvettes with a protein concentration of 5 μM. CD spectra were measured every 0.5 °C at a wavelength of 222 nm, unless otherwise specified. The reversibility of the thermal transition was checked by recording a new scan upon decreasing the temperature and by another scan after cooling of the thermally unfolded protein sample.

Table 1: Identification of HRPs from Jurkat T-Cell Lysates

HRPs	N-terminal sequence[a]	M_r	identity
HRP60	IPLDPVAGYKEPAXXD	60 000	fetuin (bovine)
HRP45	EPAVYFKEQFLDGXG	45 000	carleticulin
HRP40	KVFKXXLNXH	40 000	?
HRP30	SEEDV(?)KX	30 000	?
HRP25	XDXAXETXXKXQXA	25 000	?
HRP20	not done	20 000	α-synuclein[b]

[a] (X) No amino acid detected; (?) uncertain amino acid. [b] Identified by Western blotting method (Figure 1C).

RESULTS

Identification of Heat-Resistant Proteins from Jurkat T-Cells. To investigate the thermal behavior of proteins in a systematic way, we first searched heat-resistant proteins from Jurkat T-cells. Jurkat cell lysates were boiled in a boiling water bath, and the supernatant was analyzed on an SDS−polyacrylamide gel (Figure 1A). Although most proteins were aggregated by heat treatment, about 20 wt % of total proteins appeared to be heat-resistant. We further analyzed these heat-resistant proteins, focusing on the major protein bands identified in Figure 1A. Heat-treated Jurkat cell lysates were partially purified on an anion-exchange column, and four peak fractions (peaks I−IV), eluted by NaCl step gradients of 0.1, 0.2, 0.3, and 0.5 M, respectively, were separated on a SDS−polyacrylamide gel (Figure 1B). Seven major protein bands (HRP60, 45, 40, 30, 25, 20, and 16) labeled in Figure 1B according to the relative molecular weights were further analyzed in this study. Interestingly, most of the HRPs were eluted by high concentrations of NaCl, suggesting that the HRPs are highly acidic proteins.

We next identified the HRPs by N-terminal amino acid sequencing. The N-terminal amino acid sequences and the relative molecular weights of HRP60 and HRP45 were consistent with those of bovine serum fetuin (BSF) and human carleticulin, respectively (Table 1). BSF must have originated from the cell culture medium, which contains a large amount of fetal bovine serum. The N-terminal amino acid sequences of HRP40, 30, and 25 were not informative enough to clearly reveal the identity of the proteins (Table 1). Having the knowledge that α-synuclein, a presynaptic acidic protein of 19 kDa, is very heat-stable and is expressed in hematopoietic cells as well as in neuronal cells (*20, 26, 27*), we tested whether the HRP20 is α-synuclein. To identify the HRP20, peak I and II fractions were separated on an SDS−polyacrylamide gel and the proteins were detected with anti-α-synuclein antibodies. Immunoblot analysis results shown in Figure 1C clearly indicate that the HRP20 is α-synuclein (Table 1), an intrinsically unstructured protein (*21, 28*).

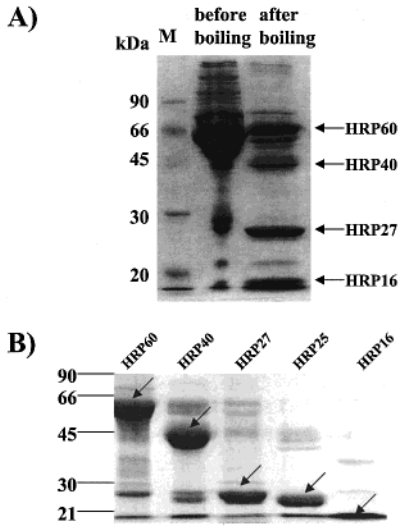

FIGURE 2: Heat-resistant proteins of human serum albumin. (A) SDS−PAGE analysis of human serum before and after heat treatment. (B) Partial purification of HRPs from human serum. Major HRPs were partially purified from human serum by DEAE anion-exchange chromatography and gel-filtration chromatography.

Table 2: Identification of HRPs from Human Serum

HRPs	N-terminal sequence[a]	M_r	identity
HRP60	APHGPGLEYRQPNXXD	60 000	fetuin (human)
HRP40	no result	40 000	?
HRP27	DEPXQSPWDRVKDLA	27 000	apolipoprotein A-I
HRP25	DEPXQSPWDRVKDLA	25 000	proteolyzed apoA-I
HRP16	GPTGTGESKXPLMVK	16 000	transthyretin

[a] (X) No amino acid detected.

Identification of HRPs from Human Serum. Since the HRP60 turned out to be BSF, we also searched for heat-resistant proteins from human serum. Similarly, human serum was boiled in a boiling water bath, and the supernatant was analyzed on an SDS−polyacrylamide gel. As shown in Figure 2A, many proteins of human serum appeared to be heat-resistant (about 70 wt % of total proteins). Using the conventional chromatography techniques, we partially purified five major HRPs (HRP60, HRP45, HRP27, HRP25, and HRP16) from human serum (Figure 2B). We observed that HRP25 was generated during the purification steps, probably due to proteolysis of another HRP. Partially purified HRPs were subjected to N-terminal amino acid sequencing to identify the nature of the HRPs. The N-terminal amino acid sequences of HRPs are shown in Table 2. As expected, the N-terminal amino acid sequence of HRP60 was consistent with that of human serum fetuin (HSF, also called human α_2-HS glycoprotein). The N-terminal amino acid sequences and the relative molecular weights of HRP27 and HRP16 are consistent with those of apolipoprotein A-I (apoA-I) and transthyretin (also called prealbumin), respectively. The N-terminal amino acid sequence of HRP25 was identical to that of HRP27, indicating that HRP25 is a proteolytic fragment of apoA-I.

Purification of HRPs of Human. We next purified the identified HRPs to investigate the structural features and conformational stabilities of the HRPs as a function of temperature. Recombinant protein of α-synuclein was purified to homogeneity by taking advantage of the heat stability of the protein and by conventional column chromatography

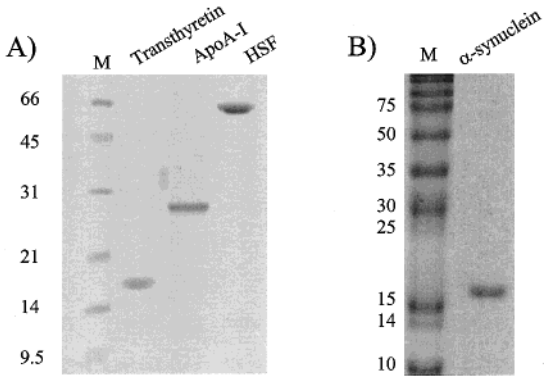

FIGURE 3: SDS−PAGE analysis of purified HRPs. (A) Purified HRPs from human serum. Proteins were analyzed on an SDS−12% polyacrylamide gel, and the proteins were stained with Coomassie brilliant blue R-250. (B) Purified α-synuclein was analyzed on an SDS−15% polyacrylamide gel, and the protein was stained with Coomassie brilliant blue R-250.

techniques (Figure 3B, lane 2), as described previously (*21*, *22*). It is well-documented that α-synuclein produced by this method is chemically and conformationally indistinguishable from α-synuclein purified without heat treatment (*22*, *28*). Other HRPs were purified from human serum without introduction of the boiling step. HSF was purified from human serum by conventional column chromatography techniques (Figure 3A, lane 4) as described previously (*23*). ApoA-I was purified as described previously (*24*) with slight modification (Figure 3A, lane 3). Transthyretin was purified from human serum by ammonium sulfate fractionation and conventional column chromatography techniques (Figure 3A, lane 2) as described previously (*25*). The HRPs used in this study were highly purified ones as determined by SDS−PAGE (Figure 3).

Structural Features and Heat Stabilities of HRPs. Secondary structural features of the purified HRPs and conformational changes of the proteins induced by the increase of temperature were investigated by circular dichroism (CD) spectroscopy in the far-UV region. To quantify thermal stability of the proteins, T_m was measured by monitoring CD signals as a function of temperature. Reversibility of the heat-induced conformational changes was also checked by temperature scanning in increasing and decreasing modes. Consequently, four major types of thermal behavior were observed as described in following examples of HRPs (group I−IV HRPs).

Group I HRPs: α-Synuclein and α_s-Casein. The CD spectrum of α-synuclein (Figure 4A) indicates that the protein almost completely lacks secondary structural elements. The 208 and 222 nm absorption bands characteristics of α-helical polypeptides and the 215 nm band of β-stranded polypeptides are absent in this spectrum. The strong absorption band at 199 nm (Figure 4A) is characteristic of random-coiled polypeptides. Fitting of the spectrum according to the method of Yang et al. (*29*) yields a secondary structure composition of 67% random coil, 23% turn, and 10% helix or sheet. Thermal unfolding of α-synuclein was also monitored by CD spectroscopy at 222 nm. Consistent with the CD spectrum, a linear temperature dependence of the CD signal, often seen with unfolded peptides, was observed (Figure 4A). Interestingly, the temperature-induced conformational change of α-synuclein was reversible as well as

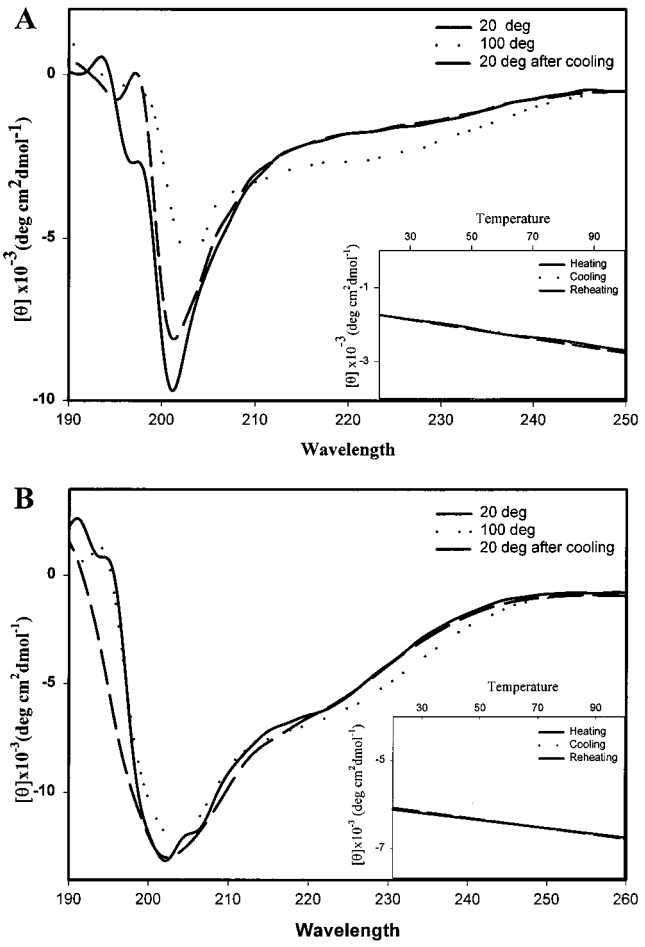

FIGURE 4: Far-UV CD spectra and melting curves of group I HRPs: (A**)** α-synuclein and **(**B) α_s-casein. Far-UV CD spectra measured at 20 and 100 °C are shown as solid and dotted lines, respectively. Dashed lines represent the spectra measured just after cooling of protein solution from 100 to 20 °C. Insets present mean molar ellipticity per residue of each protein at 222 nm as a function of temperature. Solid and dotted lines represent temperature scans from 20 to 100 °C (heating mode) and 100 to 20 °C (cooling mode), respectively. Repeated temperature scans from 20 to 100 °C are given by dashed lines.

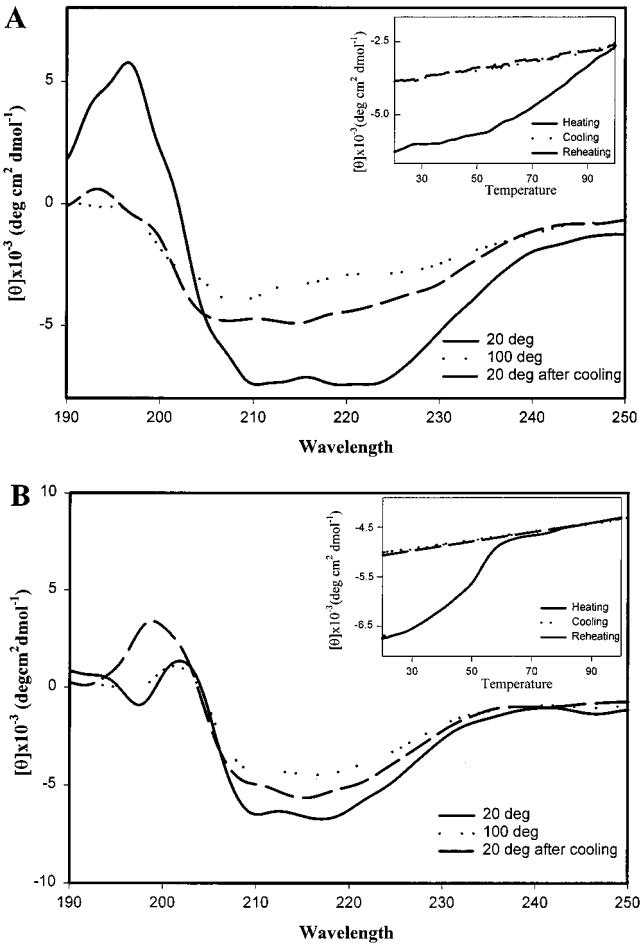

FIGURE 5: Far-UV CD spectra and melting curves of group II HRPs: (A**)** HSF and **(**B**)** apoA-I. Far-UV spectra and melting curves are presented as in Figure 4.

noncooperative. The CD spectrum of α-synuclein at 100 °C is slightly different from that at 20 °C, but it also represents the characteristics of a random-coiled polypeptide (Figure 4A). To investigate more on the proteins mainly composed of random coil, we also investigated CD spectra and thermal behavior of α_s-casein. The CD spectra and thermal behavior of α_s-casein were very similar to those of α-synuclein (Figure 4B), indicating that casein is a member of the intrinsically unstructured protein family (reviewed in refs *30* and *31*). A previous report has suggested that intrinsically unstructured proteins are generally heat-stable (ref *28* and references therein).

Group II HRPs: HSF and ApoA-I. Far-UV CD spectra of HSF are presented in Figure 5A. Spectra were measured at 20 °C (solid line), 100 °C (dotted line), and 20 °C just after cooling after the heating run (dashed line). The CD spectrum of HSF at room temperature indicates that HSF is primarily composed of α-helices. The CD spectrum was diminished at 100 °C but the overall shape was not changed, suggesting that heating does not lead to the complete unfolding of HSF. After cooling, however, the far-UV CD spectrum becomes distinguishable from the initial one, suggesting that the conformation of HSF may be irreversibly changed. However, CD spectra in the far-UV region indicate that the heat-treated HSF still retains most, if not all, of the native secondary structure. To confirm the conformational changes of HSF induced by heating, melting curves of HSF were measured. The temperature-induced changes in ellipticity at 222 nm are presented in Figure 5A. The transition midpoint appeared at 70 °C (solid line). However, the temperature course for HSF was shown to be irreversible (dotted line) and the repetition temperature scan shows the absence of any cooperative changes in ellipticity at 222 nm (dashed line). Taken together, the data obtained by CD measurements confirm irreversibility of HSF denaturation induced by the increase of temperature.

CD spectra of apoA-I at various temperatures indicate that the protein is also primarily composed of α-helices and that the secondary structure is gradually disrupted as the temperature is increased (Figure 5B). Like the case of HSF, a significant amount of the CD signal was present even at 100 °C, suggesting that heating does not lead to the complete unfolding of apoA-I. After cooling, the far-UV CD spectrum became distinguishable from the initial one, suggesting that the conformation of apoA-I may also be irreversibly changed. Melting curves of apoA-I indicate that the transition midpoint of apoA-I is 45 °C and the heat-induced conformational change of apoA-I is also irreversible (Figure 5B). Thermal behavior of bovine serum albumin (BSA) was also similar to that of HSF (data not shown)

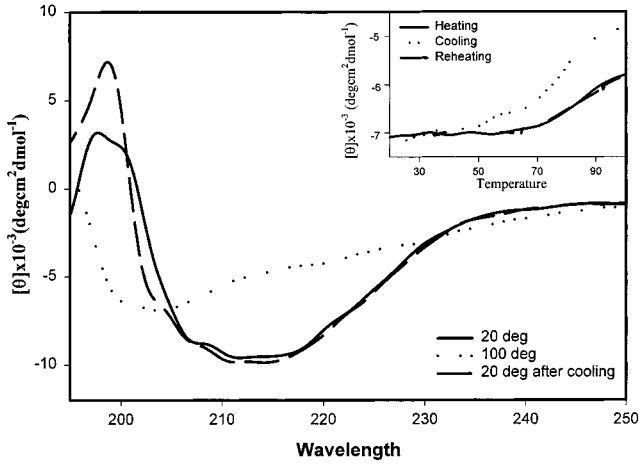

FIGURE 6: Far-UV CD spectra and melting curves of a group III HRP (transthyretin). Far-UV spectra and melting curves are presented as in Figure 4. The inset presents mean molar ellipticity per residue of the protein at 208 nm as a function of temperature. To make sure that the protein is fully denatured, the protein has been heated at 100 °C for an additional 10 min, and this process makes CD signals around 100 °C different in the thermal denaturation/renaturation curves.

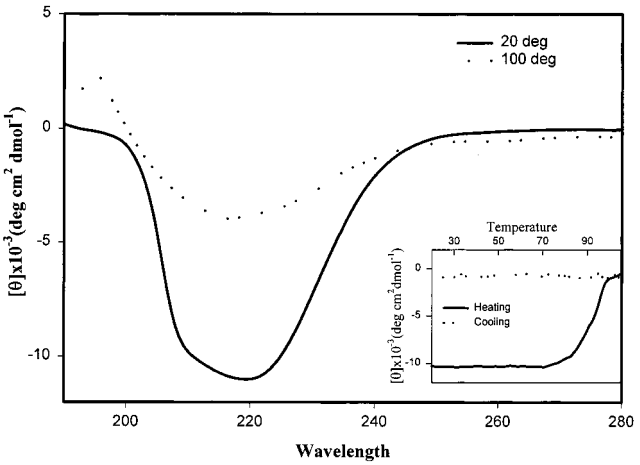

FIGURE 7: Far-UV CD spectra and melting curves of a group IV HRP (glutamate racemase). Far-UV spectra and melting curves are presented as in Figure 4.

Group III HRPs: Transthyretin. Far-UV CD spectra of transthyretin at various temperatures are presented in Figure 6. Transthyretin is a β-stranded protein (*32*). Consistent with the tertiary structure, the CD spectrum of transthyretin at room temperature shows the strong negative absorption band of 208 nm that is characteristic of β-stranded proteins (Figure 6, solid line). The CD signal of transthyretin was drastically changed as the temperature is increased (dotted line). Interestingly, however, the far-UV CD spectrum of transthyretin was completely restored after cooling (dashed line), suggesting that the heat-induced conformational change is reversible. To confirm this, melting curves of transthyretin were measured. Figure 6 represent the melting curves for transthyretin, followed by the changes in ellipticity at 208 nm. The transition midpoint appeared at 80 °C. Interestingly, the transition from native to unfolded state and from unfolded state to folded state occurred through a similar pathway. These data indicate that the thermal unfolding of transthyretin appears to be almost reversible. It was also reported that the GuHCl-induced denaturation and renaturation of transthyretin is reversible (*33*). In addition, previous works showed that the heat-induced unfolding and refolding processes of human serum albumin (HSA) and BSF are completely reversible (*34*−*36*), suggesting that reversible thermal transition is a characteristic of these HRPs.

Group IV HRPs: Hyperthermophilic Bacterial Proteins. Comparative study of the temperature effect on conformational stability of HRPs and a bacterial heat-stable protein was performed by CD. The CD spectrum of glutamate racemase (GR), a heat-stable protein from hyperthermophilic bacteria, at 20 °C (Figure 7) indicates that the protein contains well-ordered secondary structural elements. The CD spectra were not drastically changed in shape and intensity until the melting temperature. However, at 100 °C, the far-UV CD spectrum of GR was almost diminished due to the precipitation of the protein. The temperature-induced changes in ellipticity of GR at 222 nm indicates that melting temperature (T_m) of GR is 93 °C. GR was completely precipitated at 100 °C and the repetition temperature scan shows the absence of any CD signal at 222 nm, indicating that GR was irreversibly precipitated. These results shows that the bacterial heat-stable protein behaves similarly to normal heat-labile proteins as the temperature is increased, except that the heat-stable protein has a much higher T_m value. Thus, we assigned the conventional hyperthermostable proteins to group IV HRPs. Many hyperthermophilic bacterial proteins are identified to have T_m values above the boiling point, and the proteins are known to have well-ordered tertiary structures at or above 100 °C.

DISCUSSION

We have conducted a systematic investigation of thermal behavior of proteins by purifying and characterizing some heat-resistant proteins from Jurkat T-cell lysates and human serum that are not aggregated upon heat treatment. While most proteins are precipitated by heat treatment, other proteins appear to be heat-resistant. Although the fraction of heat-resistant proteins by species has not been quantified, about 20 wt % of total proteins retain solubility after boiling in a water bath. In human serum, most protein species appear to be heat-resistant (about 70 wt %), except for immunoglobulin and other major serum components. Interestingly, highly charged proteins (particularly acidic proteins) constitute a large proportion of HRPs, suggesting that solubility of protein may be related to the heat resistance of protein. We have identified five major HRPs from Jurkat T-cells and human serum by N-terminal amino acid sequencing and Western blot analysis. HRP20 and HRP45 of Jurkat T-cell lysates have been identified as α-synuclein and calreticulin, respectively, and HRP60, HRP27, and HRP16 of human serum have been identified as HSF, apoA-I, and transthyretin, respectively. α_s-casein, BSF, BSA, and HSA also appear to be heat-resistant.

We have purified the identified HRPs to homogeneity and investigated structural features and thermal behavior of these proteins by using CD spectroscopy. Our data indicate that thermal behavior of proteins can be classified into two groups, heat-labile proteins and heat-resistant proteins (HRPs). Most proteins are so heat-labile that the proteins are irreversibly denatured and precipitate upon heat treatment (Figure 8, heat-labile proteins). However, HRPs retain

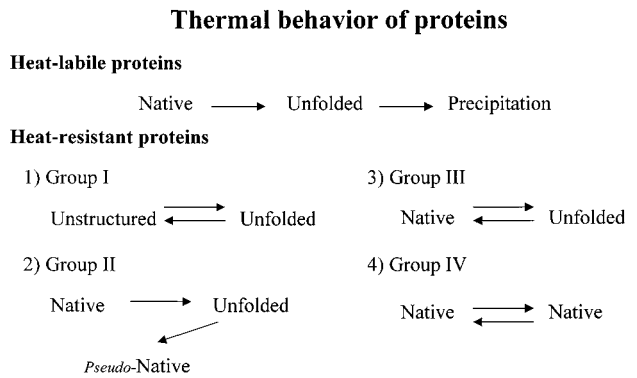

FIGURE 8: Thermal behavior of proteins. Our data indicate that the thermal behaviors of proteins can be classified into five groups. Most proteins are so heat-labile that the proteins are irreversibly denatured and precipitate upon heat treatment (heat-labile proteins). Some proteins that are intrinsically unstructured under physiological conditions constitute group I HRPs (α-synuclein and α_s-casein). Group II HRPs are represented by HSF, apoA-I, and BSA. The conformations of group II HRPs are irreversibly changed by heat treatment. Group III HRPs are characterized by a reversible conformational change upon heat treatment (transthyretin, HSA, and BSF). Group IV HRPs are characterized by the absence of conformational change upon heat treatment. No group IV HRP has been identified in human yet, but hyperthermophilic proteins, which have T_m of above 100 °C, belong to this group. Most proteins are unfolded, partially or completely, as the temperature is increased, but the renaturation processes are different from protein to protein depending on the thermodynamic stability of native (N), unfolded (U), denatured (D), and intermediate (I) states.

solubility even at the boiling temperature of water. By a systematic investigation on the unfolding and refolding process of HRPs using CD spectroscopy, we have observed four major types of thermal behavior, indicating that proteins can protect themselves through these pathways (Figure 8). Group I HRPs are represented by intrinsically unstructured proteins, such as α-synuclein and α_s-casein. Group I HRPs are heat-resistant since the proteins have a similar unfolded conformation regardless of temperature and their unfolded conformation is stable at high temperature as well as at room temperature. Group II HRPs are represented by HSF, apoA-I, and BSA; α-fetoprotein has also been shown to belong to this group (*34*). The conformations of group II HRPs are irreversibly changed by heat treatment (Figure 8). Group III HRPs are characterized by a reversible conformational change upon heat treatment (Figure 8). In this study, transthyretin appears to belong to this group. Previous studies have shown that HSA, BSF, and some small proteins behave like transthyretin upon heat treatment (*34, 35*). As in the case of normal heat-sensitive proteins, group II and III HRPs are unfolded as the temperature is increased. However, unfolded states of group II and III HRPs are stable and soluble at high temperature, while those of normal proteins are so unstable that precipitation results. Group II and III HRPs are distinguished in the renaturation process. The renatured state of group II HRPs is an irreversibly transformed conformation, while that of group III HRPs is identical to the native conformation. Group IV HRPs are characterized by the absence of conformational change upon heat treatment, since the proteins are quite stable even at the boiling temperature of water. Group IV HRPs can be categorized as conventional heat-stable proteins and hyperthermophilic bacterial proteins, which have T_m of above 100 °C, belong to this group (Figure 8). Previous works on hyperthermophilic proteins revealed that the extreme heat stability of these proteins may result from a number of subtle interactions that minimize the surface energy while maximizing packing and electrostatic interactions (*8*). In this study, no group IV HRPs have been identified in humans yet. However, if the T_m values of eukaryotic proteins are distributed according to a Gaussian profile, there might be group IV HRPs in eukaryotes. From the evolutionary viewpoint, it would be interesting to find and analyze the group IV HRPs from eukaryotes.

Thermal unfolding of the secondary structure results in extensive exposure of the polar groups involved in hydrogen bonding and increases solvent-accessible surface areas (*9*). However, the unfolding process should be investigated cautiously in the case of the proteins of highly random coil portion such as α-synuclein and α_s-casein. The free energy of α-synuclein or α_s-casein during thermal unfolding at neutral pH is expected to be very low compared to the value of −6 to −15 kcal/mol for a variety of single-domain globular proteins (*37, 38*), since most part of the proteins are already unfolded. In addition, a large portion of random coil or turns with great solvent-accessible surface area might make the proteins heat-resistant (*39*). In this respect, proteins with high portion of random coil would be interesting models to study the protein unfolding/refolding process.

The importance of the confomrationally altered state by heat is evident in the case of transthyretin. When transthyretin is jumped to 37 °C after incubation at 4 °C, amyloid fibril formation occurs to a greater extent than in a sample incubated only at 37 °C, owing to the increased fraction of monomeric state (*40*). Although the physiological relevance of the heat-induced denaturation of transthyretin is not clear, this shows that the partial denaturation of transthyretin leads to a conformationally altered monomeric intermediate that self-assembles into amyloid. Similar phenomena have been observed for fibronectin type III molecules, factor VIIa, and human immunoglobulin light chain (*41*−*43*).

Previous studies on apoA-I and BSF have revealed the molten globule-like property of these proteins (*35, 44, 45*). Specifically, the apoA-I structure is relatively weakly stabilized by tertiary interactions, and the solvent accessibility of apolar groups and the high polypeptide chain mobility observed in apoA-I appear to be important for HDL binding to apoA-I. The molten globule-like structure of apoA-I may be responsible for the low cooperativity of the thermal unfolding of lipid-free apoA-I (Figure 5B), as has been originally suggested by Gursky and Atkinson (*45*).

Hyperthermophilic proteins have extremely high T_m values (near or above boiling point) relative to their mesophilic counterparts. However, when the temperature is increased above T_m, most hyperthermophilic proteins also denature, leading to insoluble aggregation (*46*−*48*). As with other hyperthermophilic proteins, glutamate racemase from a hyperthermophilic organism appears not to change its secondary structure until the temperature is increased to T_m (Figure 7). When the temperature increases above T_m, however, it leads to the general loss of protein structure and consequently precipitation. Thus, thermal behavior of hyperthermophilic proteins is somewhat similar to that of normal globular proteins except for the high T_m value.

There are a number of examples where aggregation was attributed to the irreversible thermal unfolding (*49*). Many

studies have been performed for explaining heat stability from their primary, secondary, tertiary, and quarternary level of protein structures. However, little emphasis has been placed on the pathways of these heating processes in general (*50*, *51*). Interestingly, it has been reported that thermal stability and conformational transitions of scrapie amyloid protein correlate with infectivity (*52*). In this point, heat-resistant proteins, which are defined as proteins that are not aggregated after heat treatment, could provide some information on the origin and functional significance of thermal resistance at the molecular level. An important result, which follows from our analysis of the spectroscopic data, is that thermal resistance or stability could be achieved in several different ways. Moreover, our data suggest that reliance on the T_m alone for describing the heat stability of proteins may be inadequate in many cases. Although it is not clear at this moment that heat-induced unfolding is similar to pH- or GuHCl-induced unfolding by nature, this study could provide an interesting point in protein engineering to improve stability of proteins and to understand thermal behavior of proteins.

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