Species Differences of Serum Albumins: II. Chemical and Thermal Stability

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Purpose. The chemical and thermal stability of five species of mammalian serum albumins (human, bovine, dog, rabbit, and rat) were investigated, and conformational stabilities were compared to obtain structural information about the different albumins.

Methods. The chemical stability was estimated by using guanidine hydrochloride (GdnCl), and monitored by fluorometry and circular dichroism (CD). Thermal stability was evaluated by differential scanning calorimetry (DSC).

Results. In human, bovine, and rat albumin, two transitions were observed when GdnCl-induced denaturation was monitored fluorometrically, indicating at least one stable intermediate, although, in dog and rabbit albumin, only one transition was observed. However, GdnCl denaturation, as monitored by the ellipticity, showed a two-state transition in all species used in this study. Since these proteins, showing two transitions, contained a conserved tryptophan residue within domain II, these structural changes might have occurred in domain II during intermediate formation. DSC measurements showed that human, bovine, and rat albumin exhibited single sharp endotherms and these were clearly consistent with a two-state transition, while the deconvolution analysis of broad thermograms observed for dog and rabbit albumin showed that the absorption peaks could be approximated by a two-component composition, and were consistent with independent transitions of two different cooperative blocks.

Conclusions. These experimental results demonstrate that species differences exist with respect to the conformational stability and the mechanism of the unfolding pathway for mammalian albumin.

KEY WORDS: serum albumin; species difference; thermal denaturation; chemical denaturation; conformational stability.

INTRODUCTION

Among mammalian serum albumins, the amino acid sequences are now known for human, bovine, rat, equine, ovine, dog, and rabbit (2), and the sequence homologies are greater than 70% among these albumins. Since albumin has three homologous regions in the polypeptide chain, the three dimensional configuration is generally thought to be comprised of three homologous domains. Each domain is composed of two subdomains connected by a flexible region. These common structural motifs were very recently confirmed by detailed structural data for human and equine serum albumins, based on X-ray crystallographic data (3,4). This model clearly indicates that both interdomain and intersubdomain interactions contribute significantly to the stability of albumin molecule.

Serum albumin has not only been the subject of biochemical investigations, but has also been used as a therapeutic agent. It is used to maintain colloid osmotic blood pressure and blood pH. Recently, albumin has also been used in other therapeutic strategies as well. A major problem in the usage of serum albumin in clinical applications is viral contamination, e.g., human immunodeficiency virus, herpes, hepatitis. At present, pasteurization is generally used to eliminate such contamination. Thus, it is not only important for biochemistry research but for the pharmaceutical sciences as well to elucidate the mechanism of denaturation of the albumin molecule. However, such studies have only been carried out for bovine and human albumin by means of various spectroscopies (5–9) and differential scanning calorimetry (DSC) (10–14). In contrast, despite the fact that they are often utilized as substitutes, information on the stability and unfolding process remain unclear with respect to albumins of experimental animal species such as dog, rabbit, and rat. Comparison of the conformational stabilities of serum albumins from these species could produce useful information for the understanding of the solution properties of albumin, as well as for the engineering of a more stable human albumin by mutagenesis.

The present study describes a characterization of the thermal and guanidine hydrochloride (GdnCl) -induced denaturations of human, bovine, dog, rabbit, and rat serum albumins. The experimental results clearly indicate that considerable interspecies differences exist in the denaturation behavior of these albumins.

MATERIALS AND METHODS

Materials

Human albumin was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). Bovine, dog, rabbit, and rat serum albumins were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The samples were defatted with activated charcoal in solution at 0°C, acidified with H_2SO_4 to pH 3 and then lyophilized. All albumins used in this study gave only one band in SDS-PAGE, showing a molecular mass of approximately 66kDa. GdnCl, a specially prepared reagent, was purchased from the Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical grade.

All experiments were carried out in 67 mM sodium phosphate buffer (pH 7.4), prepared with distilled and deionized water.

Solvent-induced Denaturation of Different Serum Albumins Employing Guanidine Hydrochloride

Samples containing 1.5 mg/mL of the various albumins in different concentrations of GdnCl were incubated at various temperatures for 24-48 h prior to analysis to ensure that equilibrium had been achieved. After equilibrium had been attained, fluorescence measurements of these samples were performed with a JASCO FP-770 type fluorescence spectrometer, and circular dichroism (CD) measurements with a JASCO J-720

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type spectropolarimeter (JASCO, Tokyo, Japan). Fluorescence excitation and emission wavelengths of 295 nm and 340 nm, and an ellipticity at 222 nm were employed. Assuming a two-state behavior, the equilibrium denaturation profiles were analyzed using the linear extrapolation method as follows: the equilibrium constant for unfolding, K_D, in the presence of a denaturant may be calculated from eq. 1,

$$K_D = \frac{f_N - f_{obs}}{f_{obs} - f_D} \quad (1)$$

where f_{obs} is the observed fluorescence or ellipticities and f_N and f_D, calculated from the respective baselines before and after denaturation, are the values of the native and denatured forms of the protein, as the transition corresponds to a two-state process. Further, ΔG is also expressed by using the value of K_D (eq 2),

$$\Delta G = -RTlnK_D \quad (2)$$

where R is the gas constant, and T is the absolute temperature. Thus, since it has been found that the free energy of unfolding of proteins, ΔG, in the presence of guanidine hydrochloride is linearly related to the concentration of guanidine hydrochloride ($[GdnCl]$), ΔG_{H2O} and m were calculated by plotting ΔG versus $[GdnCl]$ as shown in eq. 3,

$$\Delta G = \Delta G_{H2O} - m[GdnCl] \quad (3)$$

where ΔG_{H2O} is the apparent free energy of unfolding in the absence of denaturant, and m is the slope of the linear plots of ΔG vs. $[GdnCl]$. This denaturation process induced by GdnCl was confirmed as a reversible process, because the removal of GdnCl by dialysis from the samples showed similar intensities of fluorescence and CD as the native albumin for all species.

If a significant change in heat capacity (ΔC_p) exists between native and denatured forms, values for the apparent changes in terms of enthalpy (ΔH_{app} and entropy (ΔS_{app}) at 4°C can be estimated by the following relation (15), assuming a two-state transition.

$$\Delta G = \Delta H - T\Delta S + \Delta C_p[T - 277.15 - Tln(T/277.15)] \quad (4)$$

The values of ΔG_{H2O} with respect to temperature were fitted to eq 4 using a nonlinear least-squares program.

Thermal Denaturation of Different Serum Albumins Estimated by Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was carried out on a MicroCal MC-2 ultrasensitive DSC (MicroCal Inc., Northampton, MA) at heating rates of 1 K/min with sample concentrations of 0.1 mM. The calorimetric reversibility of the thermally induced transition was checked by reheating the protein solution in calorimetric cell after cooling from the first run, and it was confirmed that heating to above 85°C caused irreversible denaturation. The data obtained from DSC and the temperature dependence of excess molar heat capacity, C_p, were applied to nonlinear fitting algorithms to calculate the thermodynamic parameters, thermal denaturation temperature (Tm), calorimetric enthalpy (ΔH), and van't Hoff enthalpy (ΔH_v) by employing Using Origin™ scientific plotting software.

RESULTS

GdnCl-induced Unfolding of Albumins

The unfolding of human, bovine, dog, rabbit, and rat serum albumins by GdnCl was followed by changes in the intrinsic Trp fluorescence (reflecting tertiary structure) with a 340 nm emission wavelength and far-UV CD spectrum (reflecting secondary structure). The fractions of denatured (D) state of albumin molecule, calculated from fluorescence intensity and ellipticities at 222 nm are illustrated as a function of denaturant concentration (Fig. 1). As shown in Fig. 1A, the transition curves of all five albumins exhibit an apparent two-state denaturation behavior in the presence of GdnCl when the transitions were monitored by ellipticities at 222 nm. Using these data, various thermodynamic parameters on GdnCl denaturation were estimated and are listed in Table I. The apparent free energies of secondary structure in the absence of GdnCl, $\Delta G_{H2O:222}$, decreased in the following order: dog < rabbit > human < bovine > rat. The $\Delta G_{H2O:222}$ values for globular proteins are generally in the range of 5.5 to 12.5 kcal/mol (15). The $\Delta G_{H2O:222}$ of dog and rabbit albumins were within this range, while the values of the other albumins were slightly smaller

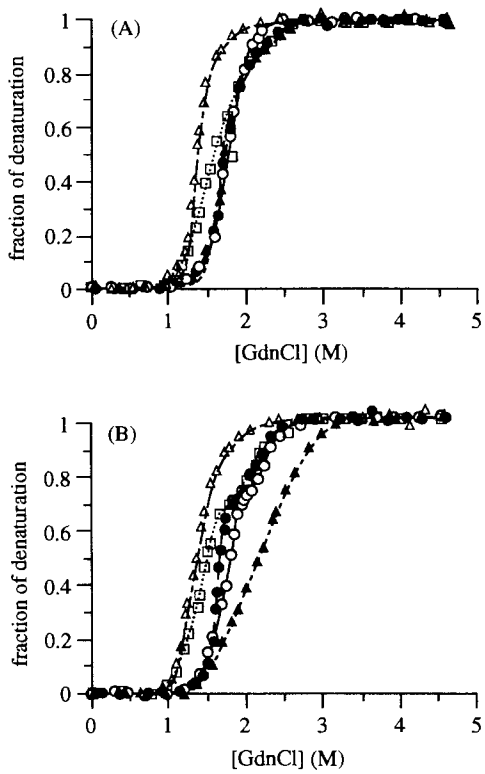

Fig. 1. Circular dichroic (A) and fluorescence (B) guanidine hydrochloride-induced denaturation profiles of albumins. Each data point represents the fraction of denaturation (F_D). Illustrated denaturation profiles of each albumin: human (○, ————), bovine (●, ----), dog (△, ————), rabbit (▲, ----------) and rat (□, ...). These experiments (n=5) were performed at pH 7.4 and 25°C.

Table I. Thermodynamic Parameters for the Guanidium Hydrochloride Denaturation of Different Serum Albumins

	CD(222nm)		Fluorescence (295-340nm)	
	m (kcal/mol/M)	ΔG_{H2O} (kcal/mol)	*m* (kcal/mol/M)	ΔG_{H2O} (kcal/mol)
human	2.84±0.96	5.03±1.81	2.71±0.03[a]	5.26±0.11[a]
bovine	2.32±0.36	3.73±0.38	2.03±0.51[a]	3.20±0.66[a]
dog	4.56±0.37	6.39±0.48	3.94±0.77	5.49±1.14
rabbit	3.28±0.20	5.62±0.50	1.66±0.09	3.43±0.39
rat	1.98±0.14	3.15±0.39	2.08±0.14[a]	3.51±0.10[a]

Note: The values represent the mean ± S.D. (n = 5).
[a] Represented as apparent values obtained from two states transition.

than 5.5 kcal/mol. As shown in Fig. 1B, GdnCl-induced denaturation of human, bovine, and rat albumins appeared to involve a two step transition when monitored by fluorescence at 340 nm. In contrast, single-step transitions were observed for dog and rabbit albumins. Unfortunately, the $\Delta G_{H2O:340}$ values which corresponded to each step in the GdnCl denaturation of human, bovine, and rat albumins could not be precisely estimated from the data shown in Fig. 1B. This could be due to the presence of an intermediate state within a narrow range of denaturant concentrations. Therefore, for these three albumins, apparent values of $\Delta G_{H2O:340}$ were approximated for the overall denaturation processes and are listed in Table I.

The values of *m*, generally accepted as the index for the extent of exposure of non-polar surfaces, were relatively large for dog albumin, and considerably small for rat albumin (Table I, CD data). This can be attributed to the solvent accessible surface area of the D state being large and the free energy of the D state being small for dog albumin, while, in the case of rat albumin, these values are opposite.

When the thermodynamic parameters of human albumin were set as standard, a good correlation coefficient (r = 0.95) was obtained between the differences in $\Delta G_{H2O:222}$ ($\Delta\Delta G_{H2O:222}$) and the *m*-value (Δm) of the albumins (Fig. 2).

Effect of Temperature on Unfolding Induced by GdnCl

Temperature dependencies of $\Delta G_{H2O:222}$ obtained from the GdnCl induced denaturation are shown in Fig. 3. Except for rat albumin, similar parabolic curves fitted with eq. 4 were observed for the remaining four albumins. These curves also exhibit characteristics similar to those observed for other globular proteins (16). That is, there were two temperature points for denaturation, the temperature at $\Delta G_{H2O:222}$ equal to 0, at a temperature lower than 0°C and a temperature point above 50°C. The temperatures at maximum ΔG_{H2O} for human, bovine, dog, and rabbit albumins (about 20°C) were similar, while that of rat albumin was relatively lower than other albumins. Such low temperature denaturation has also been observed for many proteins. This could be due to a significant contribution of the heat capacity (ΔCp) terms in eq. 4 as described in the experimental section. Thermodynamic parameters at 4°C calculated from the computed fitting of the experimental data to eq. 4 were as follows: ΔH = -29, -27, -30, -27, -1.7 (kcal/mol), ΔS = -0.12, -0.11, -0.13, -0.11, -0.02 (kcal/mol/K), ΔCp = 2.4, 2.1, 2.4, 2.0 1.1 (kcal/mol/K), for human, bovine, dog, rabbit, and rat albumins, respectively. Similar values for these three parameters were obtained for human, bovine, dog, and rabbit albumins, but not for rat albumin.

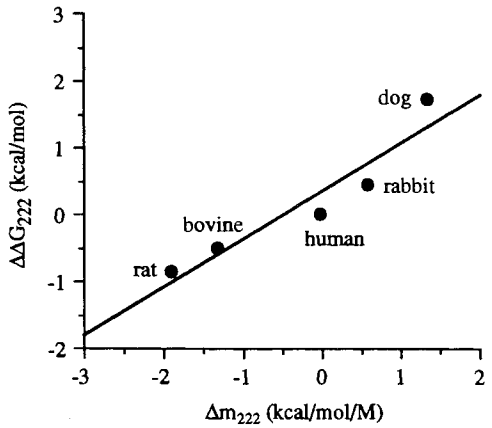

Fig. 2. Correlation between differences in stability ($\Delta\Delta G_{H2O:222}$,kcal/mol) and differences in m-value of albumins. The $\Delta\Delta G_{H2O:222}$ and Δm_{222} are expressed relative to human albumin values.

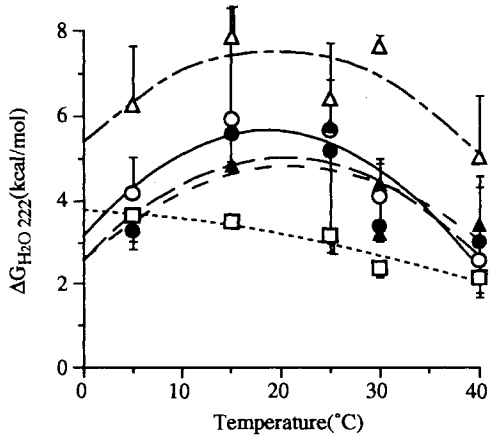

Fig. 3. Temperature dependence of the free energy during denaturation in the absence of guanidine hydrochloride. Illustrated profiles of each albumins: human (○, ————————), bovine (●, ----), dog (△,————), rabbit (▲, ----------) and rat (□, ...). These experiments (n=5) were performed at pH 7.4.

Thermal Denaturation of Albumins as Measured by DSC

The thermograms obtained from DSC of different albumins are shown in Fig. 4. Human, bovine, and rat albumins

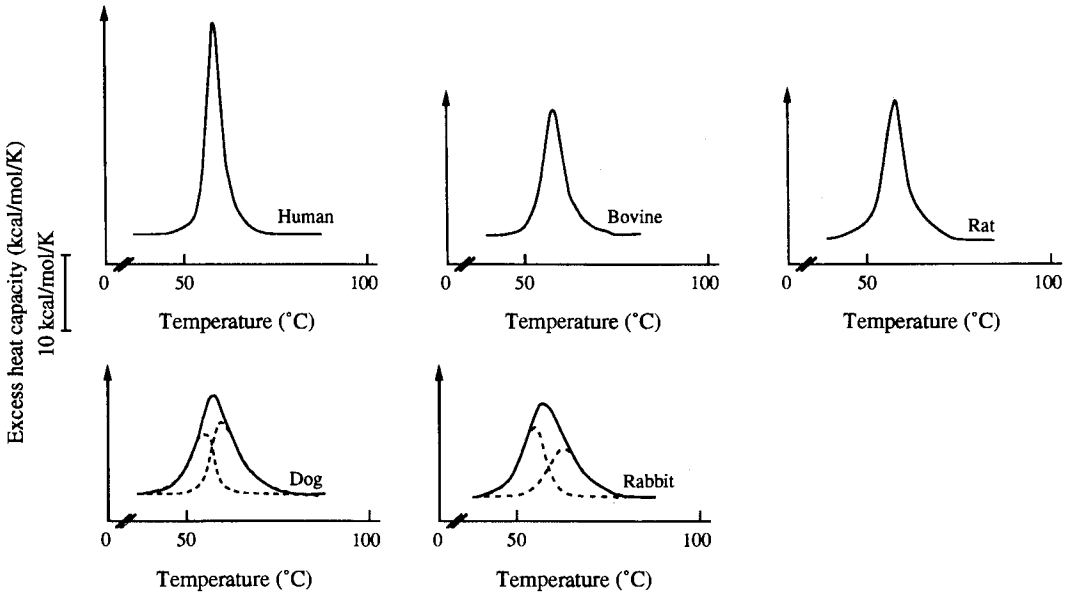

Fig. 4. Thermogram of albumins obtained from DSC experiments. Each experiment (n = 4) was carried out at pH 7.4. The broken lines represent the deconvolution analysis of the thermograms.

showed single sharp endotherms, while broad thermogram patterns were observed for dog and rabbit albumins. Deconvolution analysis indicated that the heat absorption peaks of human, bovine, and rat albumins are well explained by a single component model, while a two components model best fitted the data relative to dog and rabbit albumins, in which the lower and higher denaturation temperatures are designated *Tm1*, and *Tm2*, respectively. The thermodynamic data estimated from these thermograms are summarized in Table II. The values of *Tm* were increased in the following order: bovine < rat < rabbit < dog < human. The *Tm* value for human albumin obtained in this study was lower than those reported previously (Table II) (5,12,13,17). The order of the magnitude of the $\Delta Hcal$ value among the albumins tended to be similar to *Tm*. For the case of human albumin, the two different values of $\Delta Hcal$ are 5 cal • g^{-1} and 1.3 cal • g^{-1} based on previous reports by Ross *et al.* (11) and Picó (5). The value of $\Delta Hcal$ obtained in this study (2.5 cal • g^{-1}) fall between those two values. These discrepancies may be due to differences in experimental conditions, e.g., protein concentration, fatty acid content of human albumin, and type of microcalorimeter. The magnitude of $\Delta Hcal$ suggests that, in the D state, the tertiary structure of human albumin was not completely destroyed by thermal denaturation. The enthalpy change is generally thought to be due to the hydration of hydrophobic region, buried in native protein structure, during the unfolding process. The differences in $\Delta Hcal$ among the

Table II. Thermodynamic Parameters Obtained from DSC for Different Serum Albumins at pH7.4

Species	Tm(°C)	ΔHcal (kcal/mol)	ΔHv / ΔHcal	Ref.
human	59.65±0.05	166.3±3.2	0.69±0.01	this work
bovine	56.80±0.27	152.3±9.9	0.70±0.02	this work
dog	59.50±0.10	168.7±8.1	0.40±0.03	this work
	57.89±0.05[a]			
	60.79±0.06[b]			
rabbit	57.78±0.36	164.5±14.2	0.34±0.03	this work
	56.45±0.10[a]			
	64.56±0.11[b]			
rat	57.63±0.37	132.0±4.4	0.70±0.06	this work
human	64.6[c]	275.3	0.69	Shrake et al. (1988)
human	63.2	89.0	—	Picó (1995)
human	60.0[d]	233.3	—	Feng et al. (1994)
bovine	63.0	200.5	—	Yamasaki et al. (1990)

Note: The values represent the mean ± S.D. (n = 4). —: not reported.
[a] Represent the value of Tm1 obtained from deconvolution analysis.
[b] Represent the value of Tm2 obtained from deconvolution analysis.
[c] At pH7.
[d] The lowest transition temperature of observed three peaks.

albumins suggest that the extent of exposure of hydrophobic region caused by thermal denaturation is in the order of human, dog, rabbit > bovine > rat. The ratio of $\Delta Hv/\Delta Hcal$ is an index of the transition process to the denaturation states of proteins during thermal denaturation. The values of $\Delta Hv/\Delta Hcal$ were approximately 0.7 for human, bovine and rat albumins, 0.4 and 0.34 for dog and rabbit albumins, respectively. The values for human albumin are in good agreement with those previously reported by Shrake *et al.* (13). The values of Cp ($cal \cdot K^{-1} \cdot g^{-1}$) at 25°C were calculated to be 0.356, 0.383, 0.351, 0.361, and 0.355 for human, bovine, dog, rabbit, and rat albumins, respectively. These values are slightly larger than corresponding values reported for other globular proteins (16).

DISCUSSION

The evidence presented herein demonstrates that species differences exist for the structural stability of mammalian serum albumins. This can be, in part, attributed to the fact that recent extensive studies clearly demonstrated that the substitution of a few amino acid residues significantly influences the protein structure of the D state as well as the N state (18). Based on the present observations, the thermal and GdnCl-induced denaturation of five serum albumins may be characterized as follows: For bovine and rat albumins, the mechanism of denaturation appears to be similar to that of human albumin. The data obtained by monitoring the tryptophan fluorescence of human, bovine, and rat albumins as a function of GdnCl concentration indicate the presence of at least one intermediate conformational state during the GdnCl-induced unfolding process. A similar finding was also reported for the urea-induced structural transformations in bovine albumin and a fragment containing domain II and III (8). This finding suggests that domain I of bovine albumin is not involved in the formation of an intermediate during urea denaturation. Human and rat albumins possess only a single Trp residue, conserved in all five albumins, within domain II, while, for bovine albumin, an additional Trp residue is located in domain I. As can be seen in the crystal structure of human albumin, this conserved Trp residue (214-Trp) acts as an important stabilizer (by hydrophobic packing force) for the interface between subdomain IIA and IIIA. Accordingly, in such a region, small but significant structural changes in the three albumins appear to occur during the formation of the intermediate state. In contrast to GdnCl-induced unfolding, the thermal denaturation process can be regarded as a two-state transition, that is, in these proteins, the probability of all the intermediate states between the native and denatured ones is very low and they appear as a single cooperative system.

Unlike human albumin, broadening thermograms, possibly consisting of two components, were observed for dog and rabbit albumins. Human albumin was also observed to undergo biphasic thermal denaturation in the presence of long chain fatty acids, e.g., palmitate. Shrake and Ross explained this phenomena by a ligand redistribution model which involves uneven ligand distribution within protein species, fatty acid-poor and fatty acid-rich species, giving rise to biphasic denaturation (13). However, in this study, the ratios of albumin to fatty acid were larger than 10. Judging from the values of $\Delta Hv/\Delta Hcal$ and the fatty acid level in albumins, the thermal unfolding process of dog and rabbit albumins can more likely be explained by an independent transition of two different cooperative blocks. The crystal structures of human and equine albumins reveal a heart shaped molecule which is composed of two halves (one contains domain I and subdomain IIA, the other contains domain III and subdomain IIB) (3,4). Therefore, the two cooperative blocks which were proposed for the thermal transition of dog and rabbit albumins may correspond to the two halves of the albumin molecule. Needless to say, additional experiments on fragments of dog and rabbit albumins are called for in order to confirm such cooperative units in thermal transition.

Interestingly, there are good correlations between differences in stability $\Delta\Delta G_{H20:222}$ and deviations of *m*-value for the five albumins (Fig. 2). Since the *m*-value reflects major changes invoked in D state interactions (18), differences in the stability of albumins appear to be explained by changes in the D state structure between albumins. Judging from the *m*-value, the D state structure of dog and rabbit albumins may be the less structured state, while the D state structures of bovine and rat albumins may be more distorted in the compact denatured states than that of human albumin.

Considering the results of both thermal and GdnCl denaturation, dog albumin showed the highest stability among the five albumins, while the lowest stability was observed for rat albumin. Some of these stability differences could be caused by the differences in the D state of albumins. Recent studies have experimentally and theoretically revealed that modifications of residue hydrophobicity can alter the stability and the response to denaturants via effects on the D state ensemble (18). Therefore, future mutational studies will be required to identify which amino acid residue's hydrophobicity is the primary responder of the species differences in the stability of albumins.

Furthermore, differences in the denaturation processes, i.e., the existence of structural intermediates shown in this study indicate that functional denaturation does not occur between the N state and D state but, rather, between the N state and the intermediate state. In general, the intermediate formed during the denaturation process has a structure resembling a molten-globule or a similar structure, and this structure is known to easily aggregate. Based on these facts, in terms of stability, such an intermediate would not be a preferred structure. Furthermore, differences in the denaturation processes originate from the interdomain interactions as described above. Therefore, if the major factors for stabilities, related to both the formation of the intermediate and the interdomain interactions, are taken into consideration in designing recombinant human albumin, the development of a more stable albumin would be possible. Specifically, dog albumin, which possesses relatively different homology in terms of the amino acid sequence in domain I, showed the highest stability among all the albumins examined. Therefore, the beneficial consequences related to the stability for dog albumin will be examined and taken into consideration in the design of recombinant human albumin.

As described in "Materials and Methods," the heating of albumins to 85°C caused reversible denaturation (approximately 80–90% recovery for the native sample (data not shown)). However, heating for longer periods, such as pasteurization (at 60°C for 10-11 hours)(2) causes irreversible denaturation, even though the temperature is relatively low. For commercial albumin, pasteurization has been carried out by adding stabilizers such as caprylic acid. Therefore, the estimation of the effects

of stabilizers on the stability of albumins (including rHSA) will be necessary. Such experiments are presently under way at our laboratory.

Human albumin has been widely used as a therapeutic agent, and its market value achieved $1.1 billion in 1992 (2). In the near future, the supply of the recombinant human albumin with high stability as well as inexpensive cost will be awaited. Thus, the results obtained here will be basically useful for designing these recombinant albumins.

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