Secondary Structural Changes of Intact and Disulfide Bridges-Cleaved Human Serum Albumins in Thermal Denaturation up to 130°C – Additive Effects of Sodium Dodecyl Sulfate on the Changes

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Abstract: The secondary structural changes of human serum albumin with the intact 17 disulfide bridges (HSA) and the disulfide bridges-cleaved human serum albumin (RCM-HSA) in thermal denaturation were examined. Most of the helical structures of HSA, whose original helicity was 66%, were sharply disrupted between 50 and 100°C. However, 14% helicity remained even at 130°C. The temperature dependence of the degree of disrupted helical structures of HSA was discussed in connection with questions about a general protein denaturation model. When HSA lost the disulfide bridges, about two-thirds of the original helices were disrupted. Although the helices of RCM-HSA remaining after the cleavage of the disulfide bridges were relatively resistant against the heat treatment, the helicity changed from 22% at 25°C to 14% at 130°C. The helicity of RCM-HSA at 130°C agreed with the helicity of HSA at the same temperature, indicating that the same helical moieties of the polypeptides remained unaffected at this high temperature. The additive effects of sodium dodecyl sulfate (SDS) on the structural changes of HSA and RCM-HSA in thermal denaturation were also examined. A slight amount of SDS protected the helical structures of HSA from thermal denaturation below 80°C. Upon cooling to 25°C after heat treatment at temperatures below 70°C with the coexistence of SDS of low concentrations, the helical structures of HSA were reformed to the original level at 25°C before heating. A similar tendency was also observed after heat treatment at 80°C. In contrast, the helical structures of the RCM-HSA complexes with SDS are completely recovered upon cooling to 25°C even after heat treatment up to 100°C. Similar investigations were also carried out on bovine serum albumins which had the intact 17 disulfide bridges and lost all of the bridges.

Key words: human serum albumin, bovine serum albumin, disulfide bridges-cleaved human serum albumin, sodium dodecyl sulfate, thermal denaturation

1 INTRODUCTION

Thermal denaturation is one of the characteristic properties of proteins. However, most existing studies have discussed the thermal denaturation of proteins at temperatures much lower than 100°C. In the present work, thermal denaturation is examined over a wide temperature range up to temperatures as high as 130°C. The examination of protein denaturation below and above 100°C might provide new information on protein denaturation. The protein denaturation mechanism will be discussed on the basis of the present evidence that denaturation reactions are never in reversible equilibria. Many studies on protein-surfactant interactions have also been carried out for more than 70

years. In these studies, sodium dodecyl sulfate (SDS) has most frequently been adopted as the counterpart of proteins. In SDS denaturation at room temperature, the helical structures are partially disrupted in proteins with higher helicity, whereas they are partially formed in proteins with lower helicity¹⁻⁶⁾. However, these studies have so far been restricted to the interactions with proteins that are not affected by any other factor except the surfactant, that is, little attention has been paid to the interaction of a surfactant with a protein denatured by another factor. The present work examines the interactions of SDS with proteins denatured by heating below and above 100°C and demonstrates the protective effect of the surfactant on the

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protein structures.

Human serum albumin (HSA) is used as the main protein in the present work. HSA accounts for approximately 60% of the total protein in extracellular fluids and serves as the primary serum transport protein. It is a widely studied protein whose structure is now known down to the atomic level^{7,8)}. HSA has 17 disulfide bridges that are rather regularly located over the whole molecule to form many large and small loops⁷⁻⁹⁾. The helices formed in such loops correspond to a helicity of 45% within the original total helicity of 66% (at 25°C), according to our previous research^{4,5,10)}. When the disulfide bridges of HSA are cleaved, the protein might lose about two-thirds of the original helices with respect to the results of bovine serum albumin (BSA)¹¹⁾.

However, few studies have attempted to characterize the structural stability of HSA at high temperatures. The first purpose of this research is to examine the secondary structural changes of HSA in thermal denaturation up to 130°C and the recovery degrees of the denatured structures upon cooling. In addition, the same examination is carried out on the modified HSA (RCM-HSA), in which all of the disulfide bridges are reduced and carboxymethylated. It becomes clear that the helical structures are still maintained in both HSA and RCM-HSA even above 100°C, that is, parts of the hydrogen bonds, which construct the backbones of helical structures, are maintained at such high temperatures. The results will also show that the recovery of the denatured structure is restricted upon cooling after the heat treatment. The second purpose is to examine the additive effects of SDS on the structures of HSA and RCM-HSA denatured by heat treatment at various temperatures up to 130°C. Although the helical structures of HSA are disrupted in both the SDS denaturation and the thermal denaturation, the coexistence of SDS of low concentrations protects the structure of the protein against thermal denaturation. On the other hand, the addition of SDS induces the formation of helical structures in RCM-HSA. Thus, RCM-HSA in the SDS solution has the surfactant-induced helices as well as the helices remaining after the reduction of the disulfide bridges. The present research shows a distinct difference between these two types of helices in RCM-HSA in thermal denaturation at high temperatures. A similar comparison is also made on the intact BSA and the disulfide bridges-cleaved BSA (RCM-BSA).

2 EXPERIMENTAL PROCEDURES

HSA (A3782), BSA (A1900), and SDS (71725) were purchased from Sigma-Aldrich Co., LLC. A sodium phosphate buffer of pH 7.0 and ionic strength of 0.014^{4,5,10,11)} was used to prepare each solution for the measurements.

The reduction and carboxymethylation of disulfide bridges in HSA and BSA were carried out as follows¹²⁾. The

HSA or BSA sample was dissolved in a solution of 0.1 M phosphate buffer of pH 8.0 containing 8 M urea and L-dithiothreitol, the concentration of which was 20 times the molar equivalent of the Cys-Cys bridge. After the mixture was allowed to stand for about 2 h at 20°C, the resulting sulfhydryl groups were carboxymethylated by the addition of two molar equivalents of monoiodoacetic acid with respect to dithiothreitol, essentially according to the method of Crestfield *et al.*¹²⁾. In the carboxymethylation in the above procedure, the pH was maintained at 8.0 by adding a small amount of concentrated aqueous solution of sodium hydroxide. The reduced and carboxymethylated proteins, RCM-HSA and RCM-BSA, were separated from other reagents by dialysis against a phosphate buffer of pH 8.0 and then against a phosphate buffer of pH 7.0, which was used exclusively in the present study. The concentrations of both HSA and RCM-HSA were determined spectrophotometrically¹³⁾ using $\epsilon_{280} = 35000 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The concentration of BSA and RCM-BSA were determined¹³⁾ using $\epsilon_{280} = 44000 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Each protein concentration was adjusted to 10 μM for each experimental condition.

The circular dichroism (CD) measurements were carried out with a Jasco J-720W spectropolarimeter, using a 1.0 mm path length cell, at various temperatures up to 130°C. We used a special high-temperature cell holder system TC-700 ordered from Japan Spectroscopic Co. to heat an aqueous solution up to temperatures higher than 100°C. Nitrogen gas of 1.0 MPa was applied to this cell holder, and the temperature in the cell was measured using a thermistor sensor (Technol Seven Co., Ltd.) in each measurement¹⁴⁻¹⁷⁾. The CD spectrum was measured at a desired temperature after keeping the protein solution alone or the protein solution containing SDS at the temperature for 15 min. It has been confirmed by NMR measurements that a degradation of SDS is not induced even at 130°C¹⁴⁾. The irradiation of ultraviolet light more or less disrupts the structure of proteins^{18,19)}. Thus, the cell containing the protein solution was protected from ultraviolet light, when the temperature was varied or maintained. The helicity was estimated by the curve-fitting method of the far-ultraviolet-CD spectrum, using the reference spectra as determined by Chen *et al.*²⁰⁾ The simulation was carried out in the wavelength region 200-240 nm at 1-nm intervals^{10,11,14-17,21)}. Such an evaluation of the helicity enabled a discussion of the structural changes more quantitatively compared with the expression of the increase and decrease of the mean residue ellipticity at a specific wavelength.

3 RESULTS

The far-ultraviolet-CD spectra of the intact HSA and the intact BSA were closely similar in the buffer solution of pH 7.0 at 25°C. Both the helicities of HSA²¹⁾ and BSA¹⁰⁾ have

been estimated to be 66% by the curve-fitting of their CD spectra at 25°C. The 66% helicity of HSA is in excellent agreement with the 67% helicity of the protein determined by Carter and Ho on the basis of their X-ray crystallographic study⁸⁾. The value of 66% is used as the original helicity of HSA throughout the present work. The 66% helicity of HSA indicates that the helical structures are formed at 386 amino acid residues out of the total 585 residues of the protein molecule (585×0.66).

In the present study, the structural change of HSA was first examined in heat treatment up to temperature as high as 130°C. Figure 1 shows both the changed helicity of HSA with the increase in temperature and the recovered helicity upon cooling to 25°C from each of the higher temperatures. The helicity began to decrease upon heating above 35°C. The helicity sharply decreased between 50 and 100°C. Most of the helical structures were disrupted with the increase in temperature up to 100°C, but 14% of the helicity remained even at 130°C.

Upon cooling to 25°C only after the heat treatments below 45°C, the helicity of HSA was recovered to the original magnitude. When the HSA solution was heated up to temperatures above 50°C and then cooled to 25°C, the helicity was not completely recovered. Further, the higher the denaturation temperature was, the lower the recovered helicity was, as seen in Fig. 1. When the temperature was cooled from 130 to 25°C, the helicity was recovered from 14% to only 18%.

Figure 2(A) shows the SDS effect on the helicity of HSA upon keeping at several high temperatures. The profiles in a low SDS concentration range at 60, 70, and 80°C are enlarged in Fig. 2(B). At 25°C, the helical structures of HSA were partially disrupted in the SDS solution, and the helicity

Fig. 1 Helicity change of HSA with rise of temperature (○) and the recovered helicity upon cooling to 25°C from each elevated temperature of abscissa (●).

Fig. 2 (A) SDS effect on the helicity of HSA upon keeping at 60 (○), 70 (●), 80 (△), 90 (▲), 100 (□), 110 (■), 120 (◇), and 130°C (◆). The dotted line indicates the ordinal SDS denaturation of HSA at 25°C. (B) SDS effect on the helicity of the protein at 60 (○), 70 (●), 80°C (△) in a low concentration range of the surfactant.

ty decreased with an increase in the surfactant concentration (dotted line in these figures). Under these conditions, HSA thus suffered SDS denaturation in addition to thermal denaturation, both of which disrupt the helical structures. However, the decrease of helicity was distinctly restrained when SDS coexisted in low concentrations below 1 mM at 60, 70, and 80°C (Fig. 2(B)), that is, the helical structures of the protein were protected from the thermal disruption at these temperatures when slight amounts of SDS were present. Above these certain low concentrations of SDS, the helicity began to decrease with an increase in the SDS concentration at 60, 70, and 80°C (Fig. 2(A)), that is, the surfactant cooperated with the thermal denaturation to disrupt the helical structures. At temperatures above 90°C, the protective effect of low SDS concentrations was not

observed at all, but the decrease in the helicity was gradually restrained with an increase in the SDS concentration. This tendency was observed even at 120 and 130°C.

Figure 3(A) shows the SDS effect on the recovered helicity of HSA upon cooling to 25°C after heat treatment at several higher temperatures. The profiles in a low SDS concentration range at 60, 70, and 80°C are enlarged in Fig. 3(B). Upon cooling to 25°C after heat treatment at 60 and 70°C with the coexistence of SDS below 0.5 mM, the helicity was completely recovered to the original degree, that is, the helical structures of the protein were completely reformed (Fig. 3(B)). Similarly, the reformation of helical structures was accelerated with the coexistence of SDS of

Fig. 3 (A) SDS effect on the recovered helicity of HSA upon cooling to 25°C after heat treatments at 60 (○), 70 (●), 80 (△), 90 (▲), 100 (□), 110 (■), 120 (◇), and 130°C (◆). The dotted line indicates the ordinal SDS denaturation of HSA at 25°C. (B) SDS effect on the recovered helicity of HSA upon cooling to 25°C after heat treatments at 60 (○), 70 (●), and 80°C (△) in a low concentration range of the surfactant.

low concentrations below 1 mM upon cooling to 25°C after heat treatment at 80 and 90°C. Upon cooling after the heat treatment at temperatures above 100°C, the notable acceleration effect of low SDS concentrations was not observed, but the reformation of the helical structures was gradually promoted with an increase in the SDS concentration (Fig. 3 (A)). Upon cooling after heat treatment below 100°C with the coexistence of SDS above 3 mM, the helicity of HSA was recovered approximately to the same degree as that in the case of SDS denaturation at 25°C (dotted line in this figure). A similar tendency was observed even upon cooling from 130°C.

On the other hand, many helical structures of HSA were disrupted by the reduction of disulfide bridges. The helicity of RCM-HSA was 22% at 25°C. Figure 4 shows both the changed helicity of RCM-HSA with the increase in temperature up to 130°C and the recovered helicity upon cooling to 25°C from each elevated temperature (although the thermal effect is small on RCM-HSA, the same vertical graduation width as those in the other figures is used to show the magnitudes of the changes correlatively). The helicity of RCM-HSA slightly decreased above 50°C. Upon cooling to 25°C, the helicity of RCM-HSA was recovered to the original magnitude after heat treatment below 70°C.

Figure 5 shows the SDS effect on the helicity of RCM-HSA upon keeping at several high temperatures. At 25°C, the coexistence of SDS induced the formation of helical structures in RCM-HSA and the helicity was 39% above 7 mM SDS (dotted line in this figure). In the case of thermal denaturation with the coexistence of SDS below 50°C, the helicity of RCM-HSA increased similarly as that in the case of 25°C. The formation of helical structures was restrained above 60°C, as seen in Fig. 5. The final helicity of RCM-HSA decreased at each SDS concentration with the increase in temperature. However, the helical structures

Fig. 4 Helicity change of RCM-HSA with rise of temperature (○) and the recovered helicity upon cooling to 25°C from each elevated temperature of abscissa (●).

Fig. 5 SDS effect on the helicity of RCM-HSA upon keeping at 60(○), 80(△), 100(□), 110(■), and 130°C (◆). The dotted line indicates the ordinal SDS denaturation of RCM-HSA at 25°C.

were apparently formed with the coexistence of SDS below 110°C.

Figure 6 shows the SDS effect on the helicity of RCM-HSA upon cooling to 25°C after heat treatment at several temperatures above 100°C. The helicity was completely recovered to the same degree as that in the case of SDS denaturation at each surfactant concentration (dotted line) upon cooling to 25°C after heat treatment below 100°C. Even after heat treatment above 100°C, the helicity was almost recovered to the same degree as that in the case of SDS denaturation at each surfactant concentration upon cooling to 25°C.

The same examination was also made for RCM-BSA, which lost most of its original helical structures through the reduction of disulfide bridges. Figure 7 shows both the changed helicity of RCM-BSA with the increase in temperature up to 130°C and the recovered helicity upon cooling to 25°C from each elevated temperature (The vertical graduation is the same as that in Fig. 4). The helicity of RCM-BSA, which was 24% at 25°C, decreased to 15% at 130°C. Upon cooling to 25°C, the helicity of RCM-BSA was recovered to the original magnitude after heat treatment below 80°C. The helicity increased to 42% in the SDS solution at 25°C¹¹⁾. The effects of the addition of SDS on the decreasing profile and the recovery degree of the helicity of RCM-BSA with increasing and decreasing temperatures (not shown) were similar to those in the case of RCM-HSA.

4 DISCUSSION

In the SDS solution at room temperature, the helical structures are partially disrupted in HSA and BSA, whereas they are partially formed in RCM-HSA and RCM-BSA. In the entire molecules of HSA and BSA, 17 disulfide bridges

Fig. 6 SDS effect on the recovered helicity of RCM-HSA upon cooling to 25°C after heat treatments at 100(□), 110(■), and 130°C (◆). The dotted line indicates the ordinal SDS denaturation of RCM-HSA at 25°C. After heat treatments below 100°C, the helicity was completely recovered to the same degree as the SDS denaturation at each surfactant concentration upon cooling to 25°C.

Fig. 7 Helicity change of RCM-BSA with rise of temperature (○) and the recovered helicity upon cooling to 25°C from each elevated temperature of abscissa (●).

are rather regularly located to form many large and small loops, as first indicated by Brown⁹⁾. The helices formed in such loops possess 45% helicity^{9, 10)} within the original helicities of 66% of these proteins at 25°C. On the other hand, the helicity of HSA sharply decreases to 22% through the reduction of the disulfide bridges. The 22% helicity of RCM-HSA corresponds to helical moieties formed at 129 residues (i.e., 585×0.22). Interestingly, this 22% helicity of RCM-HSA approximately agrees with the difference between the helicity of the loops, i.e., 45% and the original total helicity, i.e., 66%. The relationship for the helices of BSA¹⁰⁾ and RCM-BSA is the same. These suggest

that the helices in the loops of HSA and BSA are predominantly disrupted upon the reduction of the disulfide bridges.

4.1 Structural changes of HSA and RCM-HSA in thermal denaturation

As seen in Fig. 1, most of the helical structures of HSA are sharply disrupted between 50 and 100°C. However, the 14% helicity remains even at 130°C, indicating that the helical moieties consisting of about 82 amino acid residues (i.e., 585×0.14) still remain at this high temperature. The authors emphasize that the disrupted degree of the original helices of HSA clearly depends on temperature. This is a fact, which has a simple but important significance, as will be discussed later. The present results also show that some ordered structure is never altered to a completely disordered structure even with heat treatment at 130°C. Parts of the hydrogen bonds, forming the backbone of helical structure of protein, seem to be maintained even at 130°C.

Against such a sharp disruption of the helical structure of HSA above 50°C, the same structure of RCM-HSA is hardly disrupted even at high temperatures, as seen in Fig. 4. The helical moieties remaining after the reduction of disulfide bridges appear likely to be relatively resistant against the heat treatment.

The helicities of both HSA and RCM-HSA are around 14% at 130°C, as mentioned above. Therefore, the helical moieties retained at high temperatures might be almost the same in both HSA and RCM-HSA. This also means that the helical moieties of HSA lost through the reduction of the disulfide bridges are included in the helical moieties disrupted at high temperatures. In other words, the helical structures in the loops of HSA are disrupted at high temperatures. The same relationship is obtainable in the comparison of BSA and RCM-BSA. This is also one of the findings of the present work.

Upon cooling to 25°C, the helicity of HSA is recovered to the original level only after heat treatment below 50°C, whereas that of RCM-HSA is recovered after heat treatment below 70°C. It is noteworthy that the recovery of helicity upon cooling after heat treatment is observed only in restricted temperature ranges for both HSA and RCM-HSA. The results indicate that the higher the denaturation temperature, the lower the recovered helicity in restricted temperature ranges. This tendency is commonly observed also for other proteins in thermal denaturation^{14–17}.

The abovementioned facts raise a question about two-state and three-state models, $N \rightleftharpoons D$ and $N \rightleftharpoons D' \rightleftharpoons D$ (N: native state, D: denatured state, D': intermediate between N and D), which have been often adopted in discussions on denaturation of proteins²². It is indispensable for these models that a constant N and a constant D exist at any stage in the denaturation. Furthermore, these models premise that the denaturation reaction between a constant

N and a constant D is reversible. However, the D states of HSA and RCM-HSA change with an increase in temperature above a particular temperature, as emphasized above. This is the same in the case of structural changes of other proteins. It is important to note that the D state, which depends on the change in the denaturing factor, is never constant not only in thermal denaturation but also in denaturation by denaturants, urea, guanidine, and surfactants. It should be noted that the D states, which are used in these models, are never constant and they necessarily change depending on the changes in the intensities of the denaturing factors for proteins. Another question also arises as to whether the D state exists under a native condition, that is, for example, at 25°C in the present thermal denaturation, and whether the N state exists under an extremely denatured condition, that is, for example, at 130°C in the present thermal denaturation. In other words, the denaturation reactions themselves are altered by changes in the intensities of the denaturing factors.

In addition, it is doubtful whether an equilibrium relationship exists in such denaturation reactions. On the other hand, the reversible nature of structure has been often focused in the refolding studies of proteins^{23, 24}. These refolding studies have been carried out by diluting concentrations of denaturants, such as urea and guanidine, by shifting the pH to a neutral region from acidic or alkaline, or by cooling after the heat treatment. Indeed, the terms of “reversible” and “reversibility” have been used in these refolding studies^{23, 24}. Also in the present work, the recovery of helicity upon cooling is observed after heating. However, the phenomenon of recovery of helicity upon cooling is clearly different from the ordinal reversibility of chemical equilibrium, that is, the reversible nature observed in the refolding studies is essentially different from the reversibility of chemical equilibrium.

The above reversible two-state or three-state reaction models seem to be forcibly introduced to interpret the denaturation of proteins, although the denaturation reactions have been understood to be in irreversible equilibria. It appears to be likely that both denaturation and renaturation are one-way reactions. In denaturation, a native structure changes to a denatured structure determined by the strength of denaturing factor without a backward reaction, whereas in renaturation upon the decline of the denaturing factor, each denatured structure changes to a less denatured structure without a backward reaction. The appearance of a new concept is expected to interpret denaturation and the structural changes of proteins. We consider it better to examine denaturation or structural changes of proteins assuming one-way reactions with no backward processes rather than assuming the reversible two-state reaction model or similar modified reaction models.

4.2 SDS effects on the structural changes at high temperatures

Many studies have been confined to the interactions of SDS with intact proteins that are not affected by any other denaturing factor¹⁻⁵. In contrast, little effort has been made to characterize the interactions of SDS with proteins that undergo another denaturation^{4, 25-29}. Although the helical structures of the intact HSA are disrupted in SDS denaturation at 25°C, a slight amount of SDS protects the helical structures of the protein from thermal denaturation at temperatures below 80°C (Fig. 2(B)). This protective behavior is observed at extremely low SDS concentrations, where the helicity is barely damaged in the ordinal SDS denaturation at 25°C (dotted line). The HSA concentration is 10 µM in the present work. The coexistence of SDS of concentrations around 200 µM, that is, the mixing molar ratio of [SDS]/[HSA] of approximately 20, is enough to give the maximal protective effects at 60 and 70°C. For example, the helicity is 38% in the absence of SDS at 70°C, but 57% helicity can be maximally maintained at this temperature with the coexistence of 0.2-1 mM SDS. The difference indicates that helical structures formed at 111 residues in calculation are protected from disruption by heating at 70°C.

Although most of the original helices of HSA are practically protected by such small amounts of SDS, all of the coexisting dodecyl sulfate (DS) ions do not bind to the protein even at low temperatures. The actual number of DS ions bound to the protein is considered to be much smaller than the mixing molar ratio at each temperature. Furthermore, the interaction of the protein with the DS ions becomes weak with the increase in temperature, that is, the increase in temperature shifts the binding equilibrium between the protein and DS ion toward the direction of dissociation. Thus, at high temperatures, only a few DS ions are assumed to be interacting with each of several helical moieties, which are dispersed over the whole HSA molecule.

A similar protective effect of SDS has been found in urea denaturation²⁵⁻²⁹ of HSA and BSA as well as in thermal denaturation of helical rich proteins, BSA¹⁴ and myoglobin¹⁶. It is clear that the protective effect is observed in the case of low SDS concentrations that are not enough to form micelle-like aggregates on the protein polypeptide. Markus et al. have interpreted the protective effect of a slight amount of DS ions on the protein structures assuming some cross-linking function of the surfactant ion^{26, 27}. They have explained that the native conformation of HSA is stabilized by a cross-linking of DS ions between a group of nonpolar residues and a positively charged residue located on different loops of the protein. In fact, such linkages of DS ions have been found in lysozyme by X-ray crystallography: the hydrophobic chains make contacts with particular hydrophobic residues, while the hydrophilic groups are salt-bridged to positively charged residues³⁰. The present observation of the protective function suggests that such cross-linked

DS ions might not separate from proteins even at high temperatures.

In the temperature range below 80°C, the protective effect disappears above 1 mM SDS and the helicity decreases with an increase in the surfactant concentration (Fig. 2(A)). At SDS concentrations above 1 mM, the DS ions are considered to form micelle-like aggregates on the protein polypeptide, and then, the ordinal SDS denaturation becomes predominant. Therefore, the SDS denaturation above 1 mM might cooperate with the thermal denaturation to disrupt the helical structures at temperatures below 80°C.

In the temperature range beyond 80°C, the distinct protective effect of SDS of low concentrations on the HSA structure does not appear. However, the decrease of the helicity is gradually restrained with an increase in the surfactant concentration in this temperature range (Fig. 2(A)). Beyond 80°C, the formation of micelle-like aggregates on the protein polypeptide gradually induces the protective effect. This suggests that the interaction between DS ions and the protein is gradually strengthened with an increase in the surfactant concentration even at high temperatures.

As mentioned above, one of characteristics of the SDS denaturation is that the helical structures are formed in proteins with lower helicity at 25°C. This helix-forming property of SDS distinctly appears in RCM-HSA (Fig. 5) and RCM-BSA (not shown) at high temperatures as well as at 25°C. Therefore, in the SDS solution, these reduced proteins contain not only the helices remaining after the reduction of disulfide bridges but also the SDS-induced helices at any temperature. The total helicity of the complex of RCM-HSA with SDS gradually decreases with the increase in temperature, as shown in Fig. 5, which shows its surfactant concentration dependences at several temperatures. Most of these decrements in the total helicities might correspond to the disruptions of the SDS-induced helices, since the helices, which remain after the reduction of the disulfide bridges, are hardly disrupted by the heat treatment (Fig. 4) as discussed above.

4.3 SDS effects on the structural changes upon cooling after heat treatment

Upon cooling to 25°C after heat treatment, the recoveries of the helicities of HSA, BSA¹⁴, RCM-HSA, and RCM-BSA are restricted in the absence of SDS. Upon cooling to 25°C after heat treatment at 70 and 80°C, the helicity of HSA is recovered only to 55 and 45%, respectively, in the absence of SDS. However, with the coexistence of SDS of low concentrations, the reformation of the helical structures of HSA is accelerated upon cooling to 25°C after heat treatment at temperatures below 90°C, as clearly shown in Fig. 3(A). In particular, upon cooling to 25°C after heat treatment at temperatures below 70°C with

the coexistence of SDS of low concentrations, the helical structures of HSA are reformed to the original level at 25°C before heating. The helicity is recovered up to 58% with the coexistence of SDS below 1 mM even after heat treatment at 80°C.

Upon cooling with the coexistence of SDS above 1 mM, the helical structures of HSA, disrupted by heating at temperatures below 90°C, can be reconstructed to the HSA-SDS complex state formed at 25°C (Fig. 3(A)). Upon cooling even after heat treatment at 120 and 130°C, the reformation of the helical structure is gradually promoted with an increase in the SDS concentration.

The acceleration of the reformation of helical structures upon cooling is more remarkable for the reduced proteins, RCM-HSA and RCM-BSA, with the coexistence of SDS. Upon cooling to 25°C after heat treatment below 100°C, the helicities of the SDS complexes of RCM-HSA (Fig. 6) and RCM-BSA (not shown) are completely recovered to the complex states formed at 25°C. This indicates that not only the SDS-induced helical structures but also the original helices (remaining after the reduction of disulfide-bridges) of RCM-HSA and RCM-BSA are completely reformed upon cooling after heat treatment below 100°C. Even after heat treatment beyond 100°C, the total helicities of the SDS complexes of the reduced proteins are almost recovered upon cooling to 25°C (Fig. 6). Since the recovery of the helical structures remaining after the reduction of disulfide-bridges is incomplete upon cooling to 25°C in the absence of SDS (Fig. 4), the SDS-induced helical structures in the reduced proteins might be completely recovered at each surfactant concentration upon cooling to 25°C even after heat treatment beyond 100°C.

5 CONCLUSION

The present thermal denaturation study has clarified that the helical moieties of HSA lost through the cleavage of disulfide bridges are included in the helical moieties disrupted by the heat treatment at high temperatures and that the same helical moieties in both HSA and RCM-HSA are retained at high temperatures. On the other hand, the present surfactant, SDS, disrupts the helical structures in some proteins, but forms the helical structures in some proteins¹⁻⁵). These two different types of structural changes are attained by the formation of micelle-like aggregates on protein polypeptides at relatively high SDS concentrations. In the present work, at high SDS concentrations, the helix-disrupting property appears for HSA at temperatures up to 80°C, while the helix-forming property appears for HSA at high temperatures above 100°C (Fig. 2 (A)). The helix-forming property distinctly appears also for RCM-HSA at high temperatures as well as at 25°C (Fig. 5). In addition, the protective effects of small amounts of

SDS are distinctly observed on the helical structures of HSA in heat treatment below 80°C (Fig. 2(B)). Interestingly, these effects promote the reformation of the helical structures of HSA upon cooling after heat treatment at temperatures up to 90°C (Fig. 3(A)).

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