Protective Effect of Gemini Surfactant on Secondary Structural Change of Bovine Serum Albumin in Thermal Denaturation up to 130°C

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Abstract: The effect of gemini surfactant, sodium dilaureamidoglutamide lysine (DLGL), on the secondary structure of bovine serum albumin (BSA) was examined at 25°C and at high temperatures up to 130°C. The helicity (66%) of the protein decreased to 53% in the DLGL solution at 25°C and it also decreased to 16% with rise of temperature. Although approximately half of the original helical structures were destroyed upon heating up to 75°C, most of the structures were maximally protected in the coexistence of 0.30mM DLGL at 75°C (the protein concentration was 0.010mM). At temperatures below 75°C, the protected helicity became maximal at such low DLGL concentrations. In the thermal denaturations above 80°C, the protective effect did not appear at low DLGL concentrations, but monotonously enlarged with the surfactant concentration. On the other hand, upon cooling to 25°C after the thermal denaturations below 75°C, the helicity was maximally recovered to about 60% in the presence of DLGL below 0.30mM. Upon cooling to 25°C from high temperatures above 85°C, the recovered helicity gradually increased with the surfactant concentration. The present novel effect, especially observed at low DLGL concentrations, might be fulfilled by the monomer ions of the gemini surfactant, since actual binding numbers of DLGL onto BSA are necessarily smaller than the mixing ratios around 30mol/mol.

Key words: circular dichroism, gemini surfactant, amphiphilic material, protein structure, protection of protein structure

1 INTRODUCTION

Recently, attention has been paid to gemini-type surfactants with plural hydrophobic and hydrophilic groups. Various solution properties of the gemini-type surfactants have been studied so far focusing on the valence of hydrophilic group, chain length of hydrophobic group, and the nature and length of spacer¹⁻¹⁰. Generally, the gemini surfactants are known to be superior to ordinal single-chain surfactants in better wetting properties, greater surface activities (more efficiency to reduce surface and interfacial tension), and so on. Now, the gemini surfactants have been used also for cosmetic products. In spite of such interest and importance, the effects of gemini surfactants on the protein structures have not been investigated yet. In the present work, sodium dilaureamidoglutamide lysine (DLGL) is adopted as a gemini surfactant. The hydrophilic moiety of this surfactant has two derivatives of amino acid, glutamic acid, connected by a spacer of another amino acid, lysine, as shown in Fig. 1. Since sur-

factants derived from amino acids are mild and biodegradable, they are also widely used for cosmetic and toiletry products¹¹. It is very interesting to know how a protein structure is influenced by these surfactants derived from amino acids.

On the other hand, many studies of interactions of proteins with ordinal ionic single-chain surfactants have been carried out for more than half a century¹²⁻¹⁵. In these studies, bovine serum albumin (BSA) has been most frequently adopted as a protein^{14,15}. It should be noted that the interactions has been investigated so far on the two component system of only protein and surfactant, that is, a target protein in the interactions has not been simultaneously affected by any other factor except for the coexisting surfactant. In the present study, the secondary structural change of BSA in the interaction with DLGL has been examined not only under ordinal condition but also under heat treatments at high temperatures up to 130°C. In the latter case, the protein is necessarily affected by the thermal denatura-

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Fig. 1 Chemical Structure of DLGL.

tion simultaneously with the additive effect of the surfactant. The results show that the protein structure can be protected in the thermal denaturation below 75°C in the coexistence of a small amount of DLGL. The effect of the gemini surfactant, DLGL, will be compared with that of a typical anionic single-chain surfactant, sodium dodecyl sulfate (SDS), which we have more recently reported^{16,17}.

2 EXPERIMENTAL

BSA (A1900) was purchased from Sigma Chemical Co.^{16,17}. DLGL was obtained from Asahi Kasei Chemicals Co. A sodium phosphate buffer of pH 7.0 and ionic strength 0.014^{18–22} was exclusively used to prepare each solution. The concentration of DLGL necessary to reduce the surface tension of the solvent by 20mN/m (this concentration is often called as C_{20}) was <0.1mM in the present buffer. The surface tension of DLGL solution had a minimum around 1.2mM, probably being close to the CMC in the buffer. The concentration of BSA was determined spectrophotometrically using $\epsilon_{280}^{23} = 4.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. The final protein concentration was adjusted to 10 μM .

CD measurements were carried out with a Jasco J-720 spectropolarimeter using a 1.0mm path length cell at various temperatures up to 130°C. We used a special high temperature cell holder system¹⁷ ordered from Japan Spectroscopic Corporation to heat an aqueous solution up to a temperature higher than 100°C. A real temperature of the solution in the cell was determined with a thermocouple detector. The CD spectrum was measured after keeping the protein solution at a desired temperature for 30 min. When the temperature was changed during the experiments or when the protein solution was kept at a certain temperature for

thermal equilibrium, the cell containing the protein solution was protected from the ultraviolet beam. This is because the irradiation of ultraviolet light disrupts the structure of protein^{24,25}. The helicity (content of α -helical structure) was estimated by the curve-fitting method of the CD spectrum, using the reference spectra as determined by Chen *et al.*²⁶. The simulation^{16,17,19–22} was carried out in the wavelength region 200–240 nm at 1-nm intervals.

3 RESULTS AND DISCUSSION

In the denaturation induced by ionic surfactants such as SDS, the helical structure is partially formed in nonhelical proteins and proteins with lower helicity, whereas it is disrupted in proteins with higher helicity^{14a,19–22}. BSA belongs to the latter. The helicity of BSA has been estimated to be 66%^{14a,19}. The helicity of 66% indicates that the helical structures are formed at 385 amino acid residues in the total residues, 583²⁷, of BSA molecule. This helicity decreases to about 50% in the solutions of ionic surfactant such as SDS at 25°C^{14a,19}. This means that the helical structures at more than 90 residues of the protein are disrupted in the surfactant solutions.

3.1 Structural change of BSA by DLGL at 25°C

The secondary structural change of BSA in the solution of a gemini surfactant was probably first examined in the present study. The change of helicity at 25°C is shown in Fig. 2 as a function of DLGL concentration. The helicity decreased with an increase of the surfactant concentration in the mM order range. Although the disrupted extent of the helical structure is slightly smaller than that in the

Fig. 2 Dependence of BSA Helicity on the DLGL Concentration at 25°C.

The scale of vertical axis was identical with those in Figs. 3 and 4 to compare with them.

SDS solution at 25°C^{14a,19}, this surfactant appears likely to be not so mild for a protein structure. Furthermore, the structure of BSA was disrupted at lower concentrations of DLGL compared with SDS^{14a,19} in the same buffer, suggesting more functional effect of this surfactant with plural hydrophobic and hydrophilic groups, as mentioned below in the comparison with SDS.

3.2 Effect of DLGL on structural change of BSA in thermal denaturation

We have recently investigated the secondary structural change of BSA in the thermal denaturation up to 130°C¹⁷. The helicity of BSA begins to decrease beyond 30°C and sharply decreases between 50 and 100°C. The helicity is 22% at 100°C and 16% at 130°C. Most of the thermal disruption of helical structures occurs in the temperature range up to 100°C¹⁷. The helicity of 16% indicates that most of the helical structures are disrupted at 130°C, but helical moieties, consisting of 93 amino acid residues in calculation (583 total residues $\times$ 0.16), are still maintained even at this temperature. The reversibility of the protein structure is lost upon the descent of temperature after heating beyond 50°C. Upon raising temperature beyond 50°C, the higher the denaturation temperature is, the lower the recovered helicity is¹⁷.

The main purpose of this study is to examine the effect of DLGL on the structural change of BSA in such a thermal denaturation. Therefore, the BSA solution containing DLGL of various concentrations was heated in temperature range up to 130°C. **Figure 3** shows the helicity changes of BSA in the DLGL solutions at several temperatures up to 130°C. Some members of the series have been omitted for clarity.

Three trends of helicity change were observed. One was observed below 50°C. As can be seen in this figure, the decreasing profile of the helicity with DLGL concentration at 50°C was similar to that at 25°C without heating. This indicates that the DLGL denaturation of BSA is not so affected by temperature below 50°C. **Figure 3** shows the results at 70 and 75°C as typical examples of the second trend. The decrement of the helicity was restrained in the presence of DLGL of low concentrations. The decrement of helicity decreased with an increase of DLGL concentration in a limited low concentration range at 70 and 75°C. Approximately 60% helicity was maintained at 0.15 mM DLGL at 70°C. More than 50% helicity was maintained at 0.30 mM DLGL at 75°C. A similar trend was also observed at 55 and 60°C (not shown). After these maximal protective effects were observed at temperatures between 55 and 75°C, the protected helicities decreased with an increase in the DLGL concentration within a limited concentration range. Especially at 70 and 75°C, this trend was distinct as can be seen in **Fig. 3**. The protected helicity clearly became minimal around 1.5 mM DLGL and increased with further

Fig. 3 Dependences of BSA Helicity on the DLGL Concentration at 50 (○), 70 (●), 75 (△), 80 (▲), 90 (□), 100 (■), and 130°C (◇).

The dotted line indicates the helicity change of the protein at 25°C.

increase in the surfactant concentration. The final helicities settled around 50% at high DLGL concentrations. The third trend of helicity change was observed above 80°C, where rather large amount of helical structures were thermally disrupted. The protected helicity gradually increased with an increase of DLGL concentration, that is, the decrement of the helicity by heating became more restrained as the surfactant concentration increased.

The present gemini surfactant, DLGL, shows the protective effect on the structure of BSA suffering the thermal denaturation. The extreme protective effect at lower DLGL concentrations appeared between 55 and 75°C, whereas it did not above 80°C. The present data indicate that the maximal protective effects between 55 and 75°C are fulfilled at the DLGL/BSA mixing molar ratios of 15 and 30 mol/mol at 70 and 75°C, respectively (BSA concentration is 0.01 mM). Actual binding numbers of DLGL ion to BSA are expected to be much smaller than these mixing ratios. There was a tendency that higher DLGL concentration was required to induce the maximal protective effect as the denaturation temperature was raised in this temperature range. After the protected helicities become maximal in this temperature range, they decrease and then increase with an increase in the DLGL concentration within a limited concentration range (**Fig. 3**). Minima of the protected helicities appear around 1.5–2 mM DLGL at 70 and 75°C as seen in **Fig. 3**. Such a minimum of the protective effect is not observed in the case of SDS¹⁷. However, even a magnitude of the minimal protected helicity was appreciably larger than the helicities at these temperatures in the absence of DLGL. This indicates that the presence of DLGL brings about the protection of the protein structure to some degree at any surfactant concentration.

The protective effect beyond 80°C gradually increased with an increase of DLGL concentration, as shown in Fig. 3. Interestingly, the helicity generally tended to approach around 50% at high DLGL concentrations irrespective of temperatures between 25 and 100°C. This suggests that the final BSA-DLGL complex (the surfactant-binding-saturated-complex), the secondary structure of which corresponds to about 50% helicity, is rather stable in the heat treatment in this temperature range. A similar tendency has been observed in the effect of SDS.

3.3 Effect of DLGL on helicity recovery upon cooling

In the present study, we also examined the effect of DLGL on the recovery of the helicity upon cooling to 25°C after the heat treatment. It is already known that the secondary structure of BSA completely recovers upon cooling from temperatures below 50°C, but it does not above this temperature^{16,17}. Figure 4 shows the helicities of BSA at various concentrations of DLGL upon cooling to 25°C after the heat treatment. Upon cooling to 25°C from 50°C, the recovered helicity at each DLGL concentration was mostly the same as that adopted at 25°C before heating. Upon cooling to 25°C from temperatures between 55 and 75°C, however, the recovered helicity became maximal at low DLGL concentrations. The protective effects of low DLGL concentrations were typically observed upon cooling to 25°C from 70 and 75°C. They correspond to the same observations that the protective effects become maximal at low DLGL concentrations upon keeping high temperatures in this temperature range. Although the protective effects of low DLGL concentrations were not observed upon keeping temperature at 80°C in Fig. 3, the recovered helicity became maximal around 1.5 mM DLGL upon cooling to 25°C from this temperature, as seen in Fig. 4. In this case, much amount of DLGL was required to induce the maximal recovery of helicity compared with 0.30mM required upon cooling from 75°C. The temperature of 80°C appears to be some boundary in the interaction of BSA with DLGL. A similar boundary temperature has also been observed in the BSA-SDS interaction¹⁷. The boundary temperature region slightly shifts toward higher temperature upon cooling to 25°C in the effects of both SDS and DLGL. This might be due to a decrease of binding number of each surfactant ion to BSA with rise of temperature in addition to the disruption of helical structures by heating. Upon cooling from temperatures below 80°C, the recovered helicity became maximal at a particular DLGL concentration and decreased with an increase in the surfactant concentration down to a magnitude around 50%. However, upon cooling from 90°C, a maximum of the recovered helicity was not observed at low DLGL concentrations. When the solution was cooled to 25°C from temperatures above 90°C, the recovered helicity gradually increased with an increase of DLGL concentration, but the final helicity became lower

Fig. 4 Dependences of BSA Helicity on the DLGL Concentration upon Cooling to 25°C from 50 (○), 70 (●), 75 (△), 80 (▲), 90 (□), 100 (■), and 130°C (◇).

The dotted line indicates the helicity change of the protein at 25°C before heating.

than those upon cooling from temperatures below 80°C, as shown in Fig. 4. Although the final recovery of helicity upon cooling from 90°C was still closed to those obtained by cooling from temperatures below 80°C, it appreciably became lower for the cases of cooling from temperatures above 100°C. Such low recoveries at high temperatures have also been observed in the effect of SDS on the BSA structure¹⁷. More than half of the helical structures of BSA are disrupted above 80°C¹⁷, and it seems that a damage above 80°C is too large-scaled to be reformed by the interaction with these surfactants. Upon suffering to such high temperatures, the bound DLGL ions appear to be rearranged on BSA, forming completely different complexes between them from those formed at lower temperatures.

3.4 Comparison with SDS and feasible mechanism of interaction

The protection and the recovery of the helicity of BSA by the present DLGL with plural hydrophobic and hydrophilic groups are totally similar to those by SDS¹⁷. However, there are distinct differences between DLGL and SDS. These effects of DLGL appear in lower concentrations than those of SDS. The hydrophobicity of DLGL with two hydrophobic groups is an important factor to show a great surface activity; this surfactant has the characteristic concentration (C_{20}) below 0.1mM to reduce the surface tension of the solvent by 20 mN/m as stated in EXPERIMENTAL. Therefore, the CMC of DLGL is appreciably lower than that of SDS. This difference is essentially related to the fact that the binding of ionic surfactants to protein such as BSA generally proceeds in their free concentrations below the CMC.

On the other hand, the maximal protective effect of low SDS concentrations appears until 85°C¹⁷, whereas those of lower DLGL concentrations appears until 75°C. Upon cooling to 25°C, the maximal recovered helicity at lower SDS concentrations occurs until cooling from 90°C¹⁷, whereas the maximal protective effect of lower DLGL concentrations does until cooling from 80°C. These effects at low concentrations of SDS continue to be observed until slightly but appreciably higher temperatures compared with those of DLGL for both thermal denaturation and recovery. Related to this, we consider at present that, compared with the bulkiness of DLGL, the compactness of dodecyl sulfate ion with one hydrophobic group and one hydrophilic group appears to be effective to exhibit these effects up to higher temperatures. We have previously examined the binding isotherm to BSA of sodium bis(2-ethylhexyl) sulfosuccinate (AOT) with two hydrophobic groups and the secondary structural change of the protein by this surfactant under the same condition as the present work.²⁸ The CMC of AOT is 2.5 mM in the buffer. The helicity of BSA decreases at lower concentrations of AOT compared with SDS. The saturated binding amount of this surfactant to BSA is about 70 mol/mol²⁸ as against about 200 mol/mol of the corresponding amount of SDS determined under the same condition^{14a,18}. Both the surfactants, DLGL and AOT, with plural hydrophobic groups are superior to SDS in the effect to disrupt the helical structures of BSA, but the former two surfactants are inferior to the latter in the binding amounts to the protein. Although there are not binding data at high temperatures, we speculate that dodecyl sulfate ions more strongly interact with the protein than the surfactants with plural hydrophobic groups at high temperatures.

The protein-surfactant interactions have extensively been studied so far, but the present study is probably the first try to examine the interaction of gemini surfactant with protein. Moreover, the present examination of BSA-DLGL interaction has been made for the protein, which is simultaneously affected by another factor, thermal denaturation, as well. The thermal denaturation is one of the characteristic properties of protein. It has been understood in a qualitative sense that an original protein structure is disrupted in the thermal denaturation alone. On the other hand, the structure of BSA is disrupted in the DLGL denaturation alone (Fig. 2). However, the effect of thermal denaturation is distinctly prevented by the coexistence of DLGL. Especially, it is noteworthy that the secondary structure of the protein mostly recovers to the original state (the original helicity in the strict sense) in the presence of a small amount of DLGL upon cooling to 25°C from temperatures below 75°C. It is well discussed in the interactions of surfactant with protein or polymer that a surfactant exhibits a characteristic function by forming a micelle-like aggregate on a protein or polymer¹²⁻¹⁵. In the

present case, however, this novel effect might be fulfilled by the monomer ions of DLGL. This is because the maximal protective effect occurs at the mixing ratios of [DLGL]/[BSA] of 15 to 30 of at 70 and 75°C (Fig. 3) and the maximal recovery of the helicity appears at the mixing ratios of 10 to 30 upon cooling from 70 and 75°C (Fig. 4). These mixing ratios are calculated using the total concentrations of the surfactant below 0.3 mM for the protein concentration of 10 µM. Similar prominent effects of surfactants on the structural changes of BSA and human serum albumin (HSA) have previously been observed also at much lower mixing ratios. For example, prominent protective effects occur on the BSA structure at the mixing ratios of [SDS]/[BSA] below 6 at 50, 60, and 65°C¹⁶. At the mixing ratios of [AOT]/[HSA] of 5 to 15, similar effects have been observed on the HSA structure at 65°C²⁸. In addition, similar protection and recovery have been observed at the mixing ratios below 10mol/mol in urea denaturation^{29,30} as well. It should be noted that actual binding numbers of surfactant ion to the protein are necessarily smaller than the mixing ratios of the surfactant concentration where these prominent effects are observed. Here, there is a possibility that small micelle-like aggregates of surfactants are formed at one or two sites on the protein molecule. However, in order to induce the aforementioned effects, such aggregates would be necessary to be formed on several particular sites on the protein molecule. Even around the mixing ratio of [DLGL]/[BSA] of 30, the total surfactant concentration must be insufficient to form micelle-like aggregates separately on several particular sites of the protein molecule. Then, the present effects are considered to be fulfilled by the monomer ions of DLGL: the protein structure might be stabilized by a cross-linking function of the anionic surfactant ions between a group of nonpolar residues and a positively charged residue in the proteins. This concept, introduced by Markus *et al.*^{31,32}, has been verified later by the following two facts. (1) The X-ray crystallographic study of lysozyme-SDS system has demonstrated that the hydrophobic chain of the surfactant contacts with particular hydrophobic residues and that the hydrophilic group of the same surfactant forms salt-bridges with charged residues³³. (2) The present protective effect of ionic surfactant on the protein structure is influenced by the hydrophobic chain length and a positive or negative charge of hydrophilic group of surfactant (a negative charge of the hydrophilic group is needed in this effect on BSA, since a cationic surfactant does not protect the protein structure at all)¹⁶.

4 CONCLUSION

It is found that the gemini surfactant, DLGL, acts to pro-

tect the BSA structure in a monomer state in a similar manner to an ordinal anionic surfactant, SDS. The effect of the surfactant on the secondary structure of BSA distinctly differs below and above a boundary temperature around 80°C. Further studies on another protein are in progress and will be reported in due course. These phenomena have been overlooked in many studies of mixed systems of only surfactant and protein. Such a protective effect can be observed only in the interactions of surfactant with protein that is simultaneously influenced by another factor. These examinations might clarify characteristic properties of surfactant as well as protein. The present protective effect is due to both the hydrophobic nature and hydrophilic nature of the anionic surfactant ion used in this work. This information suggests a possibility that amphiphilic materials such as a phospholipid might act not only to form vesicles but also to affect structures and functions of proteins as characteristic and strong amphiphilic monomers.

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