

REVIEW

Kinetic Aspects of Surfactant-Induced Structural Changes of Proteins—Unsolved Problems of Two-State Model for Protein Denaturation—

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Abstract: The kinetic mechanism of surfactant-induced protein denaturation is discussed on the basis of not only stopped-flow kinetic data but also the changes of protein helicities caused by the surfactants and the discontinuous mobility changes of surfactant-protein complexes. For example, the α -helical structures of bovine serum albumin (BSA) are partially disrupted due to the addition of sodium dodecyl sulfate (SDS). Formation of SDS-BSA complex can lead to only four complex types with specific mobilities depending on the surfactant concentration. On the other hand, the apparent rate constant of the structural change of BSA increases with an increase of SDS concentration, indicating that the rate of the structural change becomes fast as the degree of the change increases. When a certain amount of surfactant ions bind to proteins, their native structures transform directly to particular structures without passing through intermediate stages that might be induced due to the binding of fewer amounts of the surfactant ions. Furthermore, this review brings up 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 without hesitation in discussion on general denaturations of proteins. First of all, doubtful is whether any equilibrium relationship exists in such denaturation reactions. It cannot be disregarded that the D states in these models differ depending on the changes of intensities of the denaturing factors. The authors emphasize that the denaturations or the structural changes of proteins should be discussed assuming one-way reaction models with no backward processes rather than assuming the reversible two-state reaction models or similar modified reaction models.

Key words: surfactant, sodium dodecyl sulfate, protein, denaturation, kinetics

1 Introduction

Many studies on the protein-surfactant interactions have been carried out for more than half a century. Structural changes of proteins induced by the interactions have also been extensively studied¹⁻⁶. Studies on surfactant-protein systems have received attention because of their biochemical importance as well as their physicochemical interest. Sodium dodecyl sulfate (SDS) has been most frequently adopted as a representative ionic surfactant in these studies. Structural changes of proteins in denaturations induced by the representative denaturants, urea and guanidine, were also studied extensively in the 1960's and 1970's. The structural changes of proteins induced by sur-

factants such as SDS are attained in millimolar concentrations (mM , $M = \text{mol} \cdot \text{L}^{-1} = \text{mol} \cdot \text{dm}^{-3}$)¹⁻⁶), although the changes are brought about by molar concentrations of urea and guanidine. Nowadays, apart from SDS several other surfactants are used to isolate, solubilize, and manipulate proteins for subsequent biophysical and biochemical characterization. These applications skillfully depend on the amphiphilic nature of these surfactants. However, it is disregarded in the applications that these surfactants bind to proteins and more or less influence their original structures without being easily removed, and making the proteins resistant toward reversible denaturations. The phenomenon that the surfactants bound to proteins are not easily de-

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prived has an important significance for the mechanism of surfactant-induced structural changes of proteins and has been discussed in this manuscript. This review reveals that denaturations of proteins by surfactants and other denaturing factors are not easily reversible by any means.

In the ordinal surfactant-induced protein structural changes at room temperature, helical structures are partially formed in proteins with lower helicity, whereas they are disrupted in proteins with higher helicity¹⁻⁵. (The term "helicity" will be used throughout this review as a short notation for the phrase "degree of α -helical structure in protein".) However, very little is known about the kinetic aspects of the surfactant-induced structural changes of proteins. Only the present authors carried out stopped-flow measurements of the structural changes using the detections of circular dichroism (CD) and absorbance in the 1980's⁷⁻¹⁵. Thereafter, unfortunately, no investigators have attempted to study the kinetics in this field. Therefore, there are only limited kinetic data of the surfactant-induced structural changes of proteins at present.

In this review, under the existing situations, the mechanism of protein denaturation induced by surfactants is discussed on the basis of not only the kinetic data⁷⁻¹⁵ but also the changes of protein helicities caused by the surfactants^{4, 12, 16-18}, the observation of discontinuous mobility change of surfactant-protein complex^{19, 20}, and the temperature dependences of protein helicities²¹⁻²⁵, which have been studied quite recently. On the other hand, the surfactant solution is very sensitive to ionic strength. For example, the electrolyte concentration influences the critical micelle concentration (CMC) of surfactant. This well-known property is important in the study of surfactant-protein interaction since the binding of surfactant ions to a protein is completed before the equilibrium concentration of the surfactant reaches the CMC. Hence, the surfactant binding and all phenomena induced by the binding are affected by the ionic strength. Accordingly, in order to examine these phenomena in a relatively wider concentration range of surfactant below the CMC, it is required to keep the CMC at a higher concentration by avoiding the use of high concentration buffers. Hence, the present review concerning the surfactant-induced structural changes of proteins mainly refers to the related results obtained by the authors' group using an identical dilute phosphate buffer (ionic strength = 0.014, pH = 7.0) through about forty years.

This review has two purposes. One is to discuss the mechanism of denaturations of proteins and homo-polypeptides induced by surfactants on the basis of old and recent results as mentioned above. The other purpose is to bring up 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 without hesitation in discussion on general

denaturations of proteins. It cannot be disregarded that the D states in these models differ depending on the changes of intensities of denaturing factors, that is, the denaturation reactions are altered by the changes of intensities of denaturing factors. In addition, it is not clear whether an equilibrium relationship exists in such denaturation reactions. Even if these models are forcibly introduced to interpret the denaturations of proteins, the denaturation reactions would not be in reversible equilibria. This irreversibility shows a significant profile that the protein denaturation reactions are absolutely different from the ordinal chemical reactions, where the reactants and the products are related by completely reversible equilibria.

2 Kinetics of surfactant-induced structural changes of proteins and homo-polypeptides followed by stopped-flow methods

2.1 Structural changes of proteins

Kinetic studies on surfactant-induced structural changes of proteins have been neglected so far, as mentioned in Introduction. Then, there are few reports on this subject⁷⁻¹⁵. Figure 1 shows the typical CD spectra of delta-chymotrypsin in the absence (A) and the presence (B) of 20 mM SDS⁸. The positive peak at 192 nm and the negative double peaks at 206 and 222 nm observed in the difference spectrum clearly indicate the formation of α -helical structures in the protein upon the addition of SDS. Figure 2 shows representative time courses of the SDS-induced structural

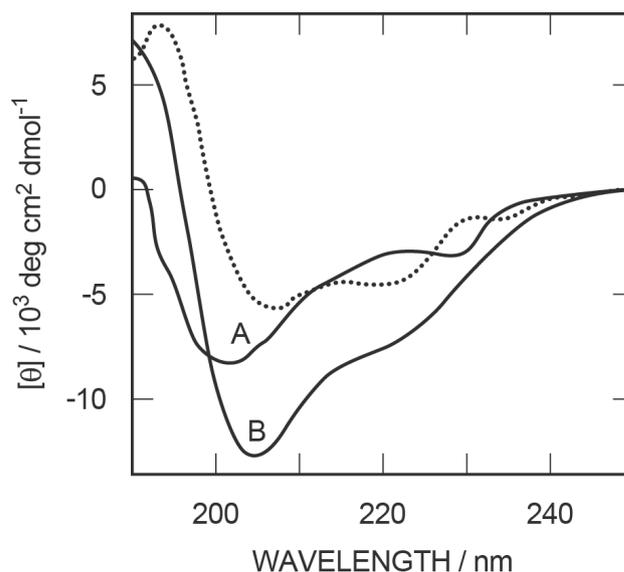


Fig. 1 Typical CD spectra of delta-chymotrypsin in the absence (A) and the presence (B) of 20 mM SDS at 25°C. The difference spectrum (dotted line) indicates that α -helical structures are formed in the protein by the addition of SDS.

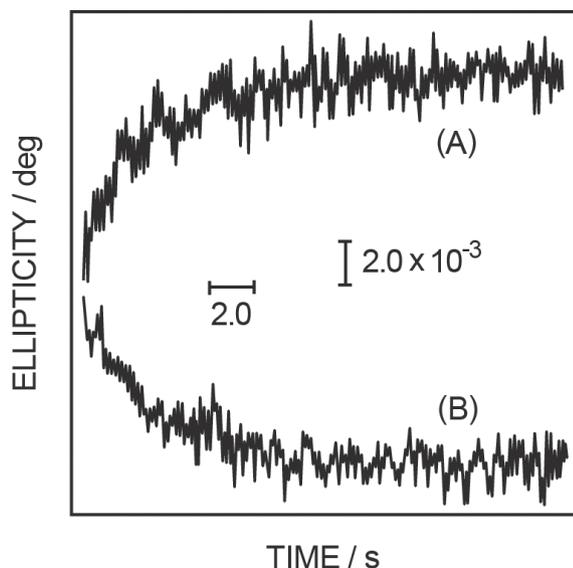


Fig. 2 Ellipticity changes of delta-chymotrypsin with time at 192 nm (A) and 206 nm (B) upon the addition of 20 mM SDS (final concentration) at 25°C. The addition of SDS was made by mixing with the stopped-flow method.

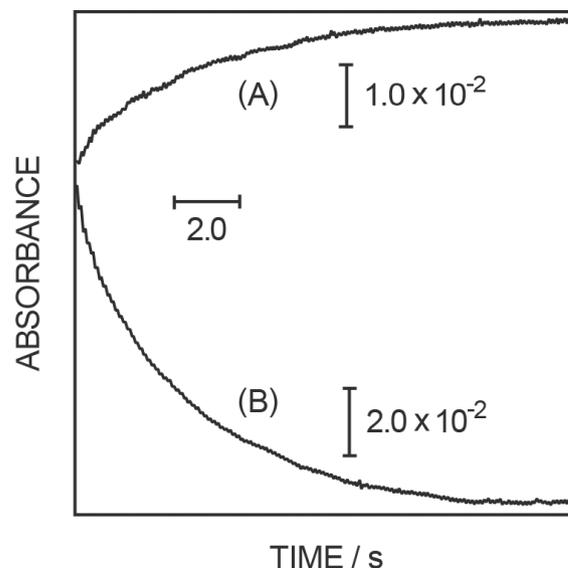


Fig. 4 Absorbance changes of delta-chymotrypsin with time at 255 nm (A) and 295 nm (B) upon the addition of 20 mM SDS (final concentration) at 25°C. The addition of SDS was made by mixing with the stopped-flow method.

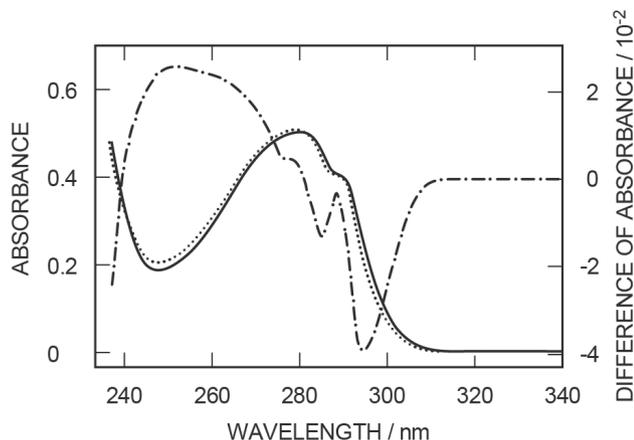


Fig. 3 Ultraviolet absorbance spectra of delta-chymotrypsin in the absence (solid curve) and the presence (dotted curve) of 20 mM SDS and the difference spectrum (dot-dashed curve) at 25°C.

change of the protein observed at these specific wavelengths by the CD stopped-flow method⁸⁾. The positive and negative magnitudes of ellipticities increase with time at 192 and 206 nm, respectively. The directions of the ellipticity changes with time correspond to the CD spectrum change from the poorly α -helical spectrum to the abundantly α -helical one, indicating that these time courses follow the coil to α -helix transition in the protein.

Figure 3 shows the effect of added SDS on the ultraviolet absorbance of delta-chymotrypsin⁸⁾. The denaturation

blue shift is observed due to the exposure of chromophores such as tryptophan and tyrosine. The absorbance change of chromophores is generally considered to reflect the tertiary structural change of protein. The positive and negative peaks appear at 255 and 295 nm, respectively, in the difference spectrum. **Figure 4** shows the representative time courses of delta-chymotrypsin at these specific wavelengths by the absorbance stopped-flow method⁸⁾. The directions of the absorbance changes with time correspond to the blue shift of the spectrum according to the SDS-induced denaturation.

Figure 5 shows the SDS concentration dependences of apparent rate constants, k , obtained from the time courses as shown in **Figs. 2** and **4**⁸⁾. The authors' original plan was to follow the secondary structural change and the tertiary structural change by the CD stopped-flow method and the absorbance stopped-flow method, respectively. However, both the CD and the absorbance stopped-flow methods yielded approximately identical values of k at each SDS concentration as seen in **Fig. 5**. Then, it appears likely that both the secondary structure and the tertiary structure of the protein simultaneously change in the same time range. It is also noteworthy that the value of k increases with an increase of SDS concentration. This indicates that the value of k increases with the progress of dodecyl sulfate (DS) ion binding with reference to the binding isotherm of SDS to the protein⁸⁾ (not shown here). Therefore, the rate of the structural change becomes fast with an increase of the degree of the change. This is the case of the protein in which the coil to α -helix transition occurs by the addition

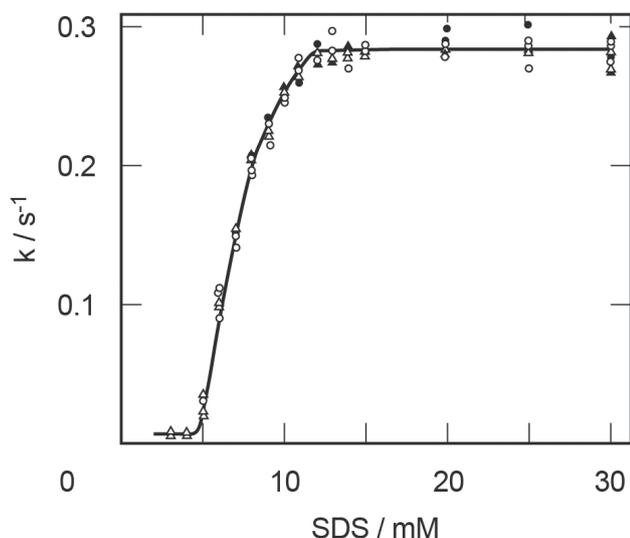


Fig. 5 SDS concentration dependences of apparent rate constants, k , for SDS-induced structural changes of delta-chymotrypsin at 25°C obtained from time courses of ellipticity changes at 195 nm (●) and 206 nm (○) by the CD stopped-flow method and the time courses of absorbance changes at 255 nm (▲) and 295 nm (△) by the absorbance stopped-flow method.

of SDS.

Bovine serum albumin (BSA) is a protein that has been most frequently studied as a counter part of SDS in the surfactant-protein interactions¹⁻⁶. The α -helical structures of BSA are partially disrupted by the addition of SDS, that is, the α -helix to coil transition occurs in the protein¹⁶. This structural change is too fast to be followed by the CD stopped flow method. Then, the structural change can be followed only by the absorbance stopped flow method¹⁰. The value of k for BSA also increases with an increase of SDS concentration as shown in Fig. 6¹⁰. In BSA, where the α -helix to coil transition occurs, the value of k increases sharply with the progress of DS ion binding, indicating that the rate becomes fast with the enlargement of the tertiary structural change of the protein. The secondary structural change is expected to occur at the same time in BSA according to the previous results of delta-chymotrypsin.

Myoglobin is one of proteins with high helicities. The α -helical structures in this protein are also partially disrupted by the addition of SDS¹². Similar kinetic tendencies of the structural changes can be observed at near-ultraviolet and Soret wavelengths of the protein¹². The α -helix to coil transition in myoglobin is considered to occur in a manner similar to the transition in BSA. The rate of the SDS-induced α -helix to coil transition is relatively faster than that of the SDS-induced coil to α -helix transition.

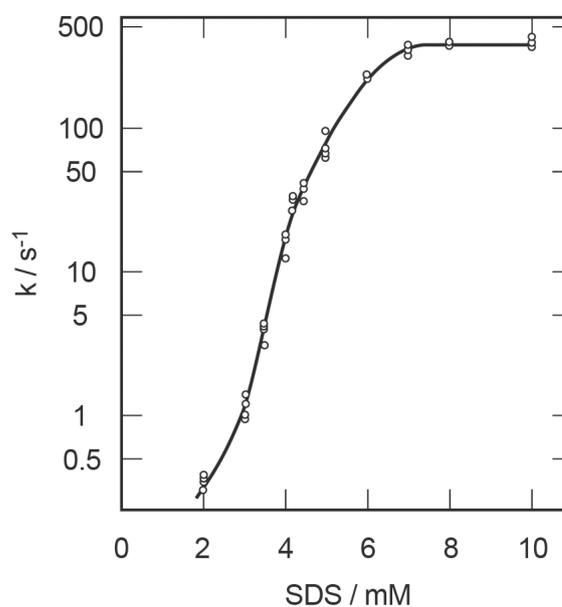


Fig. 6 SDS concentration dependence of apparent rate constant, k , for SDS-induced structural change of BSA at 25°C obtained from time courses of absorbance changes at 288 nm by the absorbance stopped-flow method.

2.2 Structural changes of homo-polypeptides

It is well known that the homo-polypeptides, poly-L-lysine (PLL)²⁶ and poly-L-glutamic acid (PLGA)²⁷ at a neutral pH are in random coil states with repulsion between the charged side chains, while they form α -helical structures upon losing their charges at alkaline and acidic pH ranges, respectively. The random coil states and the α -helical structures of these homo-polypeptides were extensively studied in connection with secondary structures of proteins in the 1960's²⁷⁻³⁰. The structural changes of these homo-polypeptides induced by surfactants have also been studied by many investigators owing to the drastic changes induced by surfactants of mM order concentrations^{13-15, 26, 31}.

Increasing concentration of SDS induces only the coil to β -structure transition in PLL²⁶, while particular concentrations of this surfactant induce the coil to α -helix transition in poly(L-ornithine)¹⁵ which is closely similar to PLL. It is interesting that the structure of PLL changes from random coil state to α -helix or β -structure depending on the concentration range of sodium octyl sulfate with shorter alkyl chain than that of SDS¹³. The authors are first and only to conduct kinetic studies on these surfactant-induced structural changes of the homo-polypeptides¹³⁻¹⁵. The kinetic studies of these structural changes can be followed only by CD stopped-flow method, because these homo-polypeptides have no near-ultraviolet absorption which is available in the absorbance stopped-flow method. The values of k of the structural changes of these homo-polypeptides also in-

crease with the surfactant concentrations (not shown), indicating that as the degrees of the structural changes become greater, their rates become faster¹³⁻¹⁵. These results are examined later together with those of the proteins.

3 Structural changes and recoveries of proteins and homo-polypeptides in thermal denaturations

3.1 Proteins

Thermal denaturation is one of the characteristic properties of protein. The original protein structure is more or less disrupted in thermal denaturation. This is also the same in urea and guanidine denaturations which are well-known as typical denaturants for proteins. The thermal denaturations and the urea and guanidine denaturations of proteins have been studied for many years.

Recently, the authors have examined the secondary structural changes of proteins in the thermal denaturation

up to 130°C. Figure 7 shows the helicity changes of four proteins with the rise of temperature and upon cooling to 25°C after heat treatments at various temperatures²¹⁻²⁵. The helicity of each protein is maintained up to 30-40°C, and decreases with the rise of temperature²¹⁻²⁵ except for lysozyme, which is not affected by heating below 60°C²⁵. These results indicate that the original structures of proteins are disrupted beyond specific temperatures. Important is that all of the hydrogen-bonds, forming the α -helical structures, are not cleaved even at 130°C. Moreover, the structural recoveries of the proteins, except lysozyme, are seen upon cooling after heating in restricted temperature ranges. Myoglobin maintains the recovery of the structural change up to a temperature as high as 75°C²⁴ (Fig. 7(B)), while lysozyme has no temperature range where the recovery is observed²⁵ (Fig. 7(C)). BSA^{21, 22} (Fig. 7(A)) and α -lactalbumin²⁵ (Fig. 7(D)) show the recovery until 45 and 60°C, respectively.

Here, the helicity, that is, the secondary structure of each protein changes beyond a critical temperature and

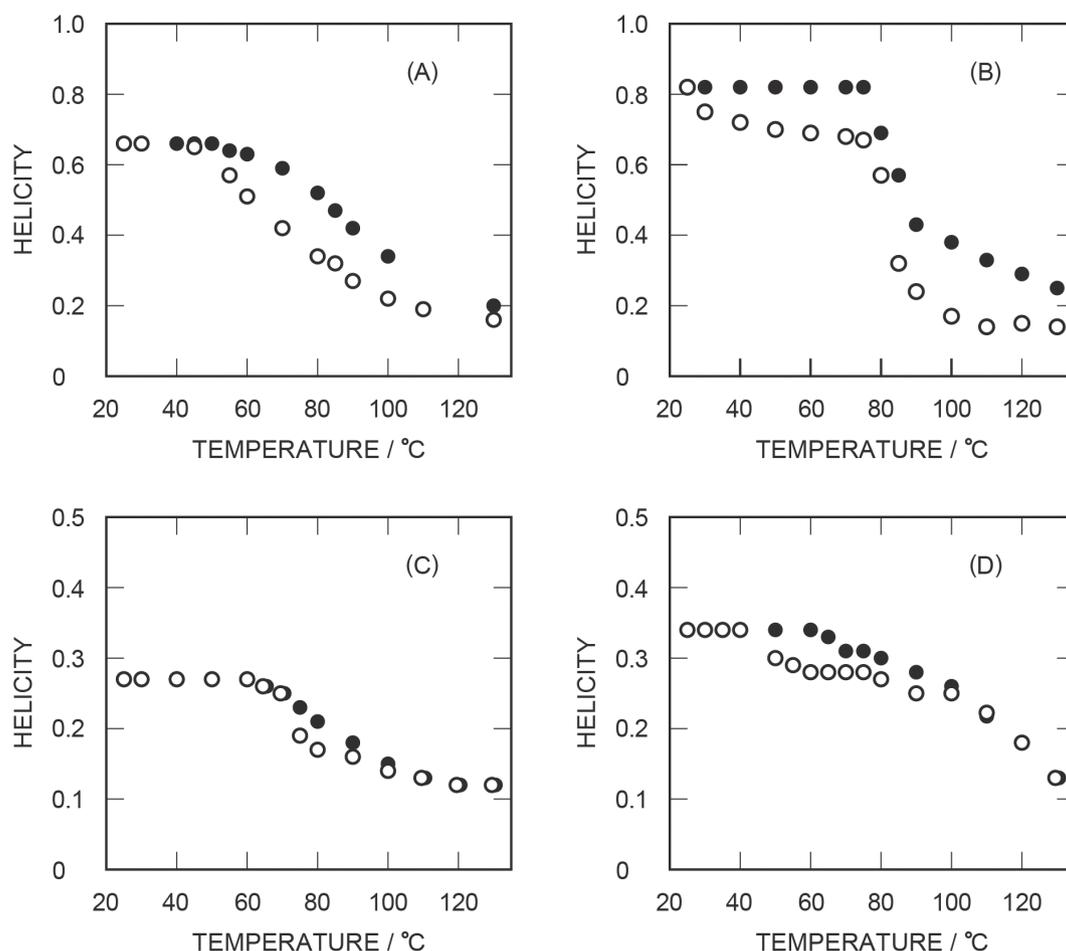


Fig. 7 Helicity changes of BSA (A), myoglobin (B), lysozyme (C), and α -lactalbumin (D) in the thermal denaturations. ○: helicity upon keeping at each temperature of abscissa; ●: helicity upon cooling to 25°C from each temperature of abscissa.

the denatured structure of protein further changes with the rise of temperature. It is emphasized here that the denatured state is altered by change of temperature and the structural recoveries of the three proteins are observed upon cooling only in particular temperature ranges.

3.2 Homo-polypeptide

Prior to the preparation of this review, the thermal stabilities of α -helical structures of PLGA and PLL were examined by means of the CD measurement. The polymerization degrees were provided to be 363 and 278 for PLGA and PLL with samples, respectively (Sigma Chemical Co.). The α -helical PLGA and PLL were prepared at pH2.8 and 12.0 adjusted with $\text{H}_3\text{PO}_4\text{-NaH}_2\text{PO}_4$ and $\text{Na}_2\text{HPO}_4\text{-Na}_3\text{PO}_4$, respectively. The residue concentrations of both the polypeptides, which were determined using the magnitudes of mean residue ellipticities, $[\theta]_{222}$, in their α -helical states ($-40000 \text{ deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ for PLGA²⁷) and $-35000 \text{ deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ for PLL^{26,28}), were adjusted to $10 \mu\text{M}$ for measurements. The CD measurements were carried out using a Jasco J-720 spectropolarimeter using a 1.0 mm path length cell. The CD spectra of PLGA were measured at various temperatures up to 140°C . On the other hand, the CD spectra of PLL were measured at temperatures up to 60°C , since this polypeptide solution became turbid beyond this temperature. In the CD measurements, we used a special high temperature cell-holder system ordered from Japan Spectroscopic Co. to heat an aqueous solution up to temperatures more than 100°C ²²⁻²⁵. A real temperature of the solution in the CD cell was determined using a thermistor sensor in each measurement. When the temperature was raised or cooled to another temperature through the measurements or when the polypeptide solutions were kept at a certain temperature, the cell containing the polypeptide solution was protected from the ultraviolet beam. This is because the irradiation of such an ultraviolet light disrupts the structures of protein polypeptides^{32,33}.

Figure 8 shows changes of CD spectra of PLGA at pH2.8 (A) and PLL at pH12.0 (B) with the rise of temperature, respectively. The spectra of both the homo-polypeptides at 25°C are typically indicative of α -helical structure. The CD spectrum of PLGA changes to the spectrum indicative of random coil with the rise of temperature. On the other hand, with the rise of temperature, the CD spectrum of PLL changes to the spectrum indicative of β -structure. The spectrum changes of PLGA and PLL agree with the general understanding: PLGA and PLL assume random coil and β -structure, respectively, upon heating²⁶⁻³⁰.

Figure 9 shows the changes of $[\theta]_{222}$ of PLGA (A) and PLL (B) with the rise of temperature and upon cooling to 25°C after heat treatments at various temperatures (temperatures on the abscissa). There are distinct different profiles at high temperatures between the α -helix to random coil transition of PLGA and the α -helix to

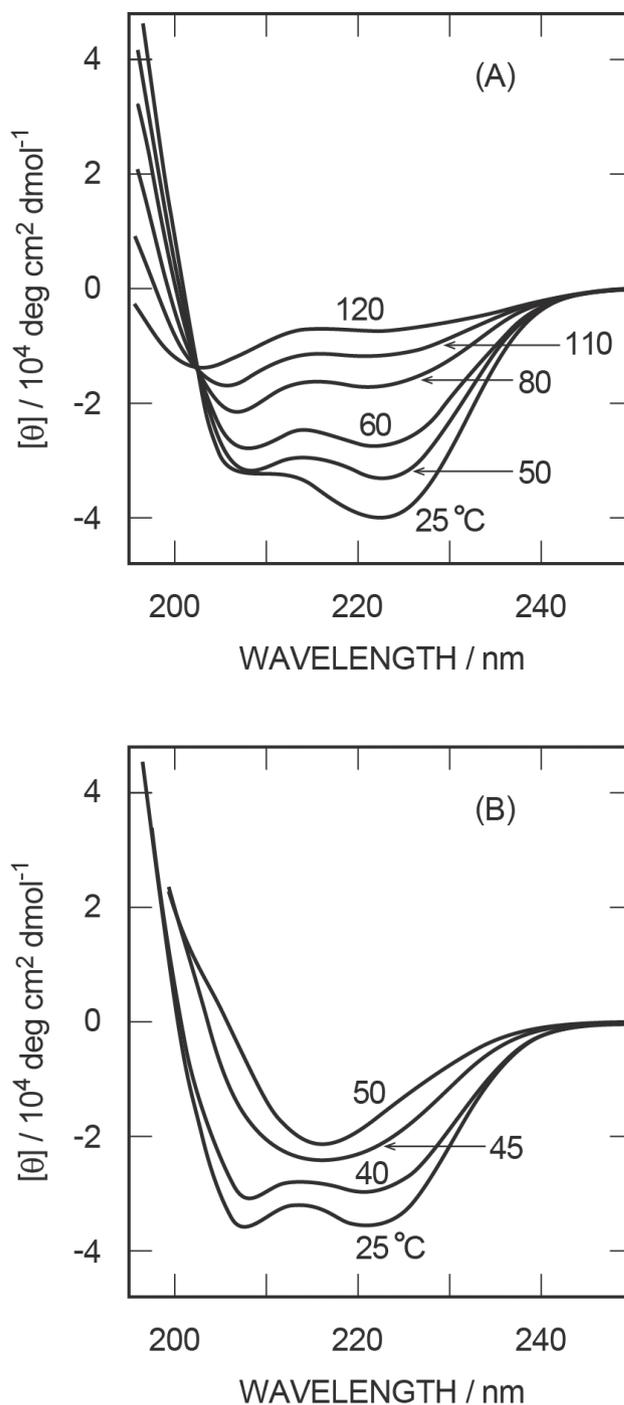


Fig. 8 Changes of CD spectra of PLGA at pH2.8 (A) and PLL at pH12.0 (B) with rise of temperature at 25°C . Numerical values indicate heating temperatures ($^\circ\text{C}$).

β -structure transition of PLL. The magnitude of $[\theta]_{222}$ of PLGA gradually decreases in a wide temperature range from 30°C up to 140°C . In contrast to this, the magnitude of $[\theta]_{222}$ of PLL abruptly decreases in a narrow temperature range between 40 and 50°C . The α -helical structures

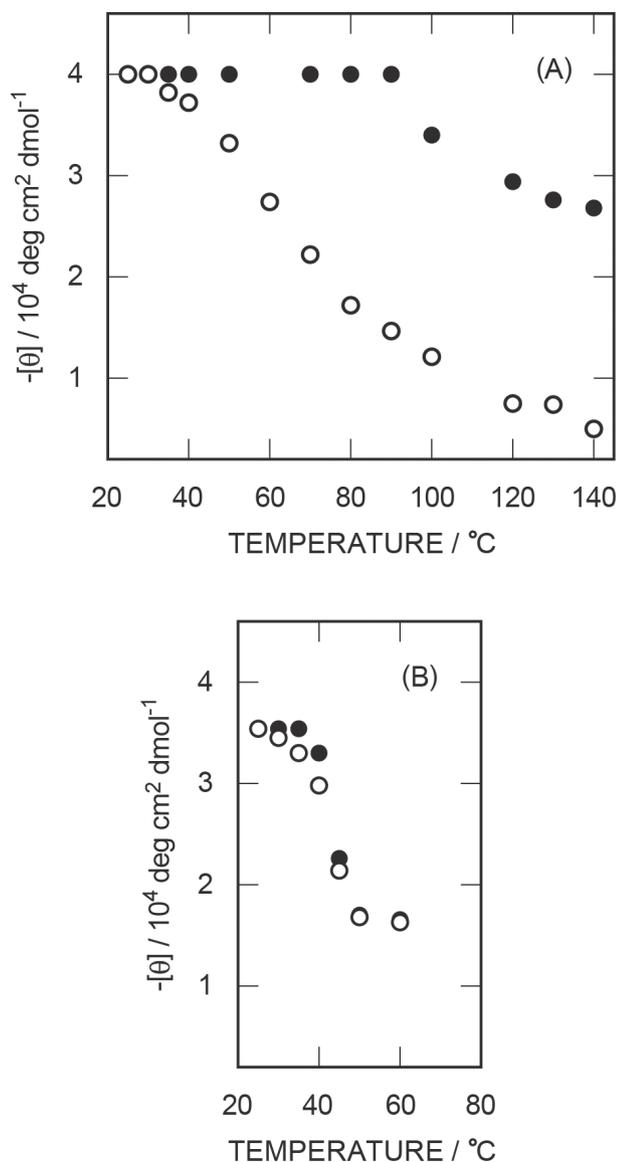


Fig. 9 Changes of $[\theta]_{222}$ of PLGA (A) and PLL (B) in the thermal denaturations. ○: $[\theta]_{222}$ upon keeping at each temperature of abscissa; ●: $[\theta]_{222}$ upon cooling to 25°C from each temperature of abscissa. The measurements were not made for PLL beyond 60°C because of the turbidity beyond this temperature.

of PLGA and PLL are converted to random coil and β -structure, respectively, as anticipated from the changes of CD spectra in Fig. 8. Distinct difference between the two homo-polypeptides also appears upon cooling to 25°C after the heat treatments. Upon cooling after the heat treatments below 90°C, the decreased magnitude of $[\theta]_{222}$ of PLGA recovers to the original value, that is, the disrupted α -helical structures of PLGA are perfectly reformed upon cooling to 25°C after heating below this temperature.

Even above 100°C, the decreased magnitude of $[\theta]_{222}$ of PLGA is considerably recovered upon cooling to 25°C. The α -helical structures of PLGA disrupted by the heat treatment are apt to be recovered upon cooling to 25°C. On the contrary, the decreased magnitude of $[\theta]_{222}$ of PLL hardly recovers upon cooling to 25°C, as can be seen in Fig. 9. The β -structures of PLL formed by heating seem to be stable because of the inter-molecular interaction, as discussed later. The denatured states of these homo-polypeptides are also altered by change of temperature as same as in the cases of proteins.

4 Formation of surfactant-protein complex

When a water-soluble protein is dissolved in an ionic surfactant solution, the surfactant ions bind to the protein to form surfactant-protein complex. The binding phenomena in the surfactant-protein interactions were extensively investigated in the initial stages of the study. During the bindings of surfactants to proteins, the hydrophilic groups of surfactant ions initially bind to the oppositely charged residues of protein polypeptide. This type of electrostatic binding is called as the stoichiometric binding. The intrinsic binding constant and the number of stoichiometric binding site have been determined¹⁻⁵. The subsequent binding of the hydrophobic groups of surfactants cooperatively occurs with both the hydrophobic residues of protein polypeptide and the hydrophobic groups of the surfactants, the hydrophilic groups of which already bind to the protein polypeptide. The latter cooperative binding is predominant in the total amount of surfactant ions bound to the protein. The bound surfactant ions finally form a micelle-like aggregate around the protein. The authors consider that the connection between the micelle-like aggregate and the protein polypeptide by the electrostatic interaction is a key to induce a large-scaled structural change of the protein in the case of ionic surfactants. It seems that nonionic surfactants hardly induce any appreciable structural change of protein owing to absence of the electrostatic interaction occurring in the ionic surfactant-protein systems.

In the combination of SDS with BSA, approximately 200 mol DS ions bind to 1mol BSA in the saturated binding stage^{4, 34}. The saturated surfactant-protein complex has a surface charge and a property similar to those of the micelle of the same surfactant^{9, 19, 20}. However, the formation of the complex appears likely to be complicated at unsaturated binding stages in low surfactant concentrations where the equilibrium surfactant concentration is insufficient to saturate the binding (The surfactant binding occurs in its monomer state). Figure 10 shows the representative capillary electrophoresis patterns of SDS-BSA complexes (A) and several kinds of mobilities of the complexes estimated from electrophoresis patterns at various SDS con-

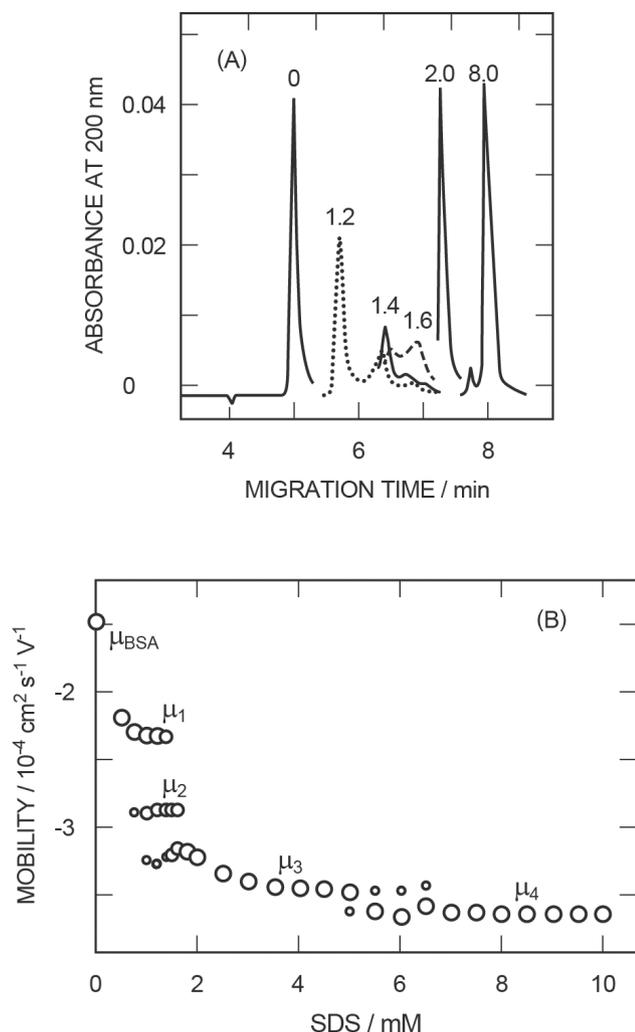


Fig. 10 Capillary electrophoresis patterns of SDS-BSA complexes at representative SDS concentrations (A) and mobilities of the complexes at various SDS concentrations (B). (A) The ordinate is absorbance of BSA at 200 nm. The abscissa is graduated for the pattern of BSA alone and the position of a small trough at 4 min in the figure corresponds to the retention time of ethanol as a reference material in the absence of SDS. The other patterns are drawn with reference to the retention time of ethanol in order to show relative changes of the retention times. Numerical values indicate total SDS concentrations (mM).

(B) μ_{BSA} : mobility of BSA alone, μ_1 to μ_4 : mobilities of SDS-BSA complexes. Three sizes of circles are used to plot different mobilities obtained at the same surfactant concentration and a circle of larger size corresponds to a mobility of more predominant species.

concentrations (B)²⁰. As seen in Fig. 10(A), a single peak can be observed in the absence of SDS and in the presence of 8 mM SDS (final concentration). The single peak in the absence of SDS gives the mobility of BSA molecule alone. The single peak at 8 mM SDS, which corresponds to the mobility of the saturated complex, indicates that only one particular type of complex is formed at the saturated binding stage. The mobilities of BSA alone and the saturated complex are indicated as μ_{BSA} and μ_4 , respectively, in Fig. 10(B). This figure also shows that intermediate complexes have mobilities of μ_1 to μ_3 . According to Fig. 10(B), the saturated complex begins to exist in the vicinity of 5 mM SDS where the intermediate complex with μ_3 is predominant. Similar tendencies were also observed by means of Tiselius type boundary electrophoresis method in the initial studies of this system^{35, 36}. The light-scattering electrophoresis method¹⁹) has also provided similar observations to those in Fig. 10(B). It should be noted that the complex formation is discontinuous and there mainly exist only the complexes of BSA with some specific numerical DS ions. Only four types of complexes with μ_1 to μ_4 are mainly formed in the SDS-BSA system according to Fig. 10(B). This is very important in the next consideration of mechanism of the surfactant-induced denaturation of protein.

5 Mechanism of surfactant-induced denaturation of protein and a question about two-state model for a general denaturation of protein

5.1 Mechanism of surfactant-induced denaturation of protein

Usually, the next expression is convenient and easy to understand the abovementioned formation of surfactant-protein complex and the accompanying structural change of protein³⁷,



where P_0 : native protein, S: surfactant ion, q, m, \cdots n: binding number of surfactant ions in each step, $P_x S_q$, $P_y S_{q+m}$, and $P_z S_{q+m+\cdots+n}$: complexes of protein with specific numerical surfactant ions. Indeed, this type of continuous multi-step reaction has been occasionally used to interpret the abovementioned complex formation. In many cases, an arrow of the backward direction is also included to indicate the reversibility in each step in Scheme 1. However, it is difficult for the authors to assume not only the reversibility of each equilibrium but also the presence of every species at every surfactant concentration as expressed by Scheme 1. Figure 11 schematically describes the assumed formation processes of surfactant-protein complexes with an increase of surfactant concentration. When the surfactant concentration is insufficient to saturate the binding, there

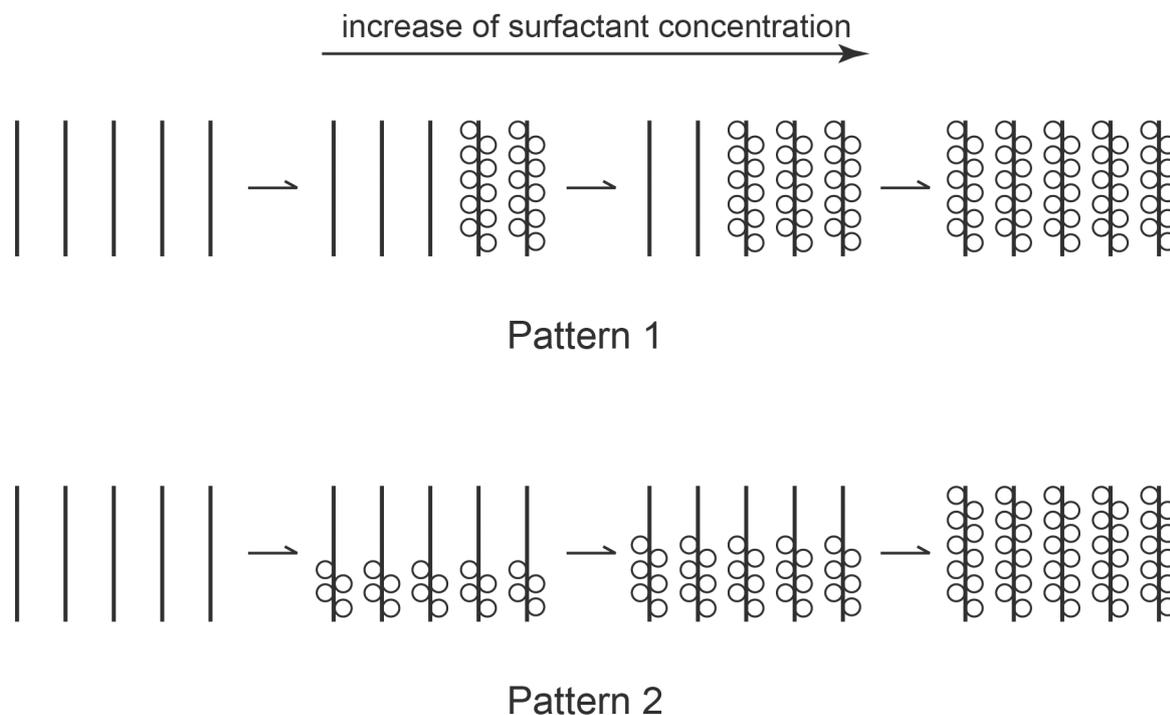


Fig. 11 Schematic drawing of surfactant-protein complex formation processes. Vertical rod: protein polypeptide, circle: surfactant ion. This drawing emphasizes a difference of binding profiles at insufficient concentrations of surfactant ions to saturate the binding (two of the center in four pieces of each). Pattern 1: the binding of insufficient surfactant ions saturates only on some of polypeptides and the amount of saturated complexes increases with an increase of surfactant concentration, Pattern 2: the binding of insufficient surfactant ions equally occurs to every polypeptide and the binding amount of surfactant ions equally increases on each polypeptide with an increase of surfactant concentration.

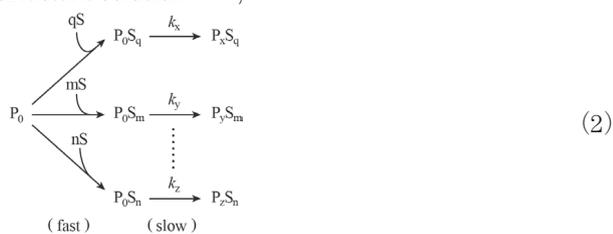
might be two possibilities: (1) the binding of surfactants occurs only to some of polypeptides and the other polypeptides remain unbound (Pattern 1 in Fig. 11); (2) the binding almost equally occurs to every polypeptide (Pattern 2 in Fig. 11). Pattern 2 emphasizes that, at insufficient surfactant concentrations (two of the center in four pieces), there is neither the native protein state drawn on the left edge nor the saturated complex state drawn on the right edge. Here, the result in Fig. 10(B) clearly indicates the presence of the complexes with specific mobilities, that is, with particular binding numbers of bound DS ions, at insufficient surfactant concentrations. Important is that there is neither the native protein (P_0) with μ_{BSA} nor the saturated complex ($P_zS_{q+m+\dots+n}$) with μ_4 at insufficient surfactant concentrations in Fig. 10(B). Further, it is difficult to remove the surfactant ions bound to protein from the protein. Although the removal of bound surfactant ions from protein solutions has been attempted in several ways, the surfactant ions bound to protein are never easily removed even after the addition of a third material³⁸⁻⁴². These facts lead to the interpretation that, at an insufficient surfactant concentration, an intermediate complex such as P_xS_q coexists neither with P_0 nor with the final

complex of $P_zS_{q+m+\dots+n}$. The result in Fig. 10(B) indicates that plural intermediate complexes really coexist only at limited insufficient surfactant concentrations, 0.8 to 1.5 mM and 5 to 6.5 mM, in the SDS-BSA system, suggesting that, if possible, the equilibria exist only between particular complexes. These facts might be enough to deny the possibility such as Pattern 1 in Fig. 11, indicating that a continuous multi-step equilibrium, as expressed by Scheme 1, should be realistically excluded.

Furthermore, the abovementioned kinetic results cannot be interpreted either by the continuous multi-step reaction model as expressed by Scheme 1. The surfactant concentration dependences of k of the structural changes of both the proteins and the homo-polypeptides indicate that their rates become faster with the progress of the surfactant binding, as explained above. This means that the greater the degrees of the structural changes are, the faster their rates become. If a structural change proceeds according to Scheme 1, the longest time is required for the transition from P_0 to the final complex of $P_zS_{q+m+\dots+n}$, which is the most denatured state. As against this, a transition from P_0 to a final species $P_zS_{q+m+\dots+n}$ is faster than a transition from P_0 to an intermediate species such as P_xS_q . Therefore, the

observed kinetic phenomena cannot be explained by the continuous multi-step reaction model such as Scheme 1.

The results in Fig. 10 clearly indicate that the binding number of surfactant ions cannot continuously increase with an increase of surfactant concentration. This means that stable complexes have limited discontinuous binding numbers of surfactant ions, that is, the binding number of surfactant ions changes stepwise depending on the surfactant concentration, as anticipated from the discontinuous change of mobility in Fig. 10(B). In the SDS-BSA system, there are only four complex states with μ_1 to μ_4 according to Fig. 10(B). Accordingly, another reaction model is required to interpret such complex formations accompanying structural changes of protein and homo-polypeptide in a surfactant solution^{5, 8, 10)},



where k_x , k_y , and k_z : rate constants and $k_x < k_y < k_z$; P_0 : native protein or homo-polypeptide (only this abbreviation will be used for a while in this section in order not to repeat a long diction of "native protein or homo-polypeptide"), P_xS_q , P_yS_m , and P_zS_n : complexes of protein with specific numerical surfactant ions. The complex state of P_zS_n in Scheme 2 corresponds to the final complex state of P_zS_q in Scheme 1. In this model, the complex formation has an individual process due to the binding amount of surfactant ions and the binding of surfactant ions to P_0 is completed before the structural change of P_0 in each step. The profile of the complex formation between surfactant and protein has been mentioned in the previous section. The complexes are formed mainly by the hydrophobic interactions of the hydrophobic groups of surfactants in bulk with hydrophobic moieties of protein polypeptide and the hydrophobic groups of surfactants which already electrostatically bind to oppositely charged residues of protein. It can be expected that surfactant ions rapidly bind to P_0 with reference to micellization kinetics of surfactants^{43, 44)}, since the binding rate of surfactant ions to P_0 seems to be of approximately the same time order as the rate of association of surfactant ions in a micelle formation (μs - ms). The abovementioned rates of the structural changes of P_0 in a surfactant solution are appreciably slow compared to those of the micelle formations of surfactants. In each complex formation process, the surfactant ions rapidly bind to P_0 before the structural change of P_0 . In other words, the structure of P_0 cannot change to a particular structure simultaneously with the binding of a certain amount of surfactant ions. After the binding of a specific amount of surfactant ions, the structure of P_0 is transformed only to a

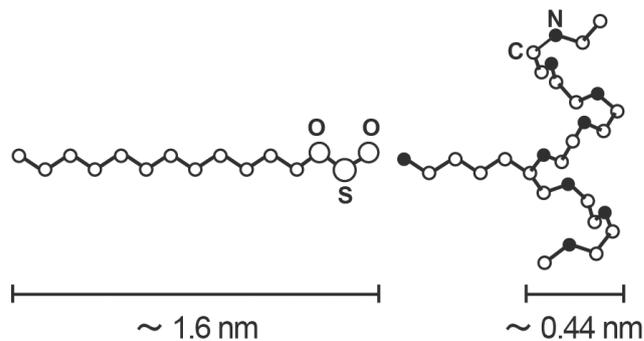


Fig. 12 Rough comparison of the bulkiness of DS ion bound to lysine residue of polypeptide with the size of α -helical backbone structure of the polypeptide.

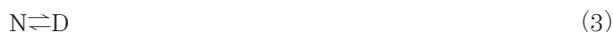
particular structure in a complex state. Then, the structures of P_0 appear to be obliged to change together with many surfactant ions bound to them. Here, Fig. 12 demonstrates the bulkiness of DS ion, especially of its hydrophobic group, against the size of α -helical backbone structure of polypeptide. This bulkiness of the surfactant ion must obstruct various delicate and fine behaviors of protein molecule. It is anticipated that the bulky hydrophobic group of DS ion, which electrostatically binds to polypeptide (Fig. 12), is interacting with many hydrophobic groups of other DS ions. The hydrophobic group of a surfactant ion, bound in this manner, is obliged to come into maximal contact with the hydrophobic groups of the other bound surfactant ions. In the case of protein, the hydrophobic interaction must also occur with hydrophobic moieties of protein polypeptide. With the progress of structural change of P_0 , the bound surfactant ions must be moved to unoccupied or more favorite binding sites. Therefore, the structural changes of P_0 induced by surfactant involve not only the structural changes of polypeptides themselves but also the rearrangement of the surfactant ions bound to them and the solvent-ordering processes around the hydrophobic groups of surfactant ions, which are occasionally exposed to an aqueous environment. The rearrangement process of the surfactant ions may take a lot of time in the complex states with fewer bound surfactant ions. On the contrary, the time of the rearrangement of bound surfactant ions might relatively become short in the saturated binding state where all of the binding sites might be occupied. As a result, the rate of the structural change would become faster with an increase of the bound surfactant ions. Such rearrangements of surfactant ions, which must take time compared to their binding, might become a rate-determining step in the structural changes of P_0 .

The structural changes of P_0 induced by surfactant must occur in the complex states with the surfactant ions, which have bulky hydrophobic groups (Fig. 12). Structural change of P_0 is fettered by bulky bound surfactant ions

with amphiphilic property. In addition, the structural change occurs without passing through any intermediate structure that might be formed by the binding of fewer amounts of surfactant ions than the amount causing the change. This is because the more the binding amount is, that is, the greater the degree of the structural change is, the faster its rate becomes, as stated above. The reaction mechanism such as **Scheme 2** might be more suitable than **Scheme 1** for the understanding of the kinetic aspect of the structural change of P_0 induced by surfactant as well as the abovementioned discontinuous complex formation between surfactant and protein observed by the electrophoresis (**Fig. 10**). **Scheme 2** insists that when a certain amount of surfactant ions bind to P_0 , the native structure directly transforms to another particular structure, that is, the binding of a certain amount of surfactant ions causes transition only to a particular structure via each individual process.

5.2 A question about two-state model for a general denaturation of protein

There is a famous two-state model for denaturation or structural change of protein, that is,



where N: native state (or native structure), D: denatured state (or disrupted structure)^{45–56}. There are also three-state model and similar modified models assuming intermediates between N and D^{57–61}. These models have been easily adopted even nowadays to examine the equilibria and kinetics of denaturations or structural changes of proteins. However, it should be noted at first that the relationship between N and D in **Scheme 3** are essentially different from the equilibrium relationship of the reactants and products in an ordinal chemical reaction such as



where A, B, C, and D represent the reaction species that more or less exist in equilibrium under various conditions. Against this, the D state in the two-state model and similar modified models naturally changes depending on the pH, temperature, and concentrations of denaturants including surfactants. In addition, the D state denatured at a certain concentration of denaturant is further influenced also by a change of the temperature. Needless to say, these phenomena have been well-recognized so far. Nevertheless, it is also fact that the two-state model and similar modified models have been used in many equilibrium and kinetic studies of denaturations or structural changes of proteins. The authors have several articles in which such data have been examined under the assumption of **Scheme 3** or its concept^{45–61}. However, we have a question about the present situation that the two-state model and similar modified models are easily treated in the same category as

an ordinal chemical reaction such as **Scheme 4**.

If we consider the structural changes of the proteins in the thermal denaturations in **Fig. 7** according to the two-state model, the decrease of helicity would mean a forward movement of equilibrium of the model, which is probably assumed at an arbitrary heating temperature. A simple problem is whether the denatured state (D) of protein exists before the rise of temperature or whether the native state (N) of protein remains at a maximal heating temperature. The results in **Fig. 7** clearly indicate that the denatured structure of protein itself changes with the rise of temperature. Also in the denaturations by urea and guanidine, the denatured structure itself changes by heat treatment. The degrees of structural changes of proteins induced by these denaturants are further increased by the rise of temperature, that is, the denatured structures by these denaturants before the rise of temperature naturally differ from those after the heat treatment. These structural changes cannot be explained under the assumption of one identical denatured state against the native state.

Subsequently, this problem is schematically examined by the simple example of the α -helix to β -structure transition of PLL (**Fig. 9**). **Figure 13** schematically describes two pathways of the α -helix to β -structure transition with the rise of temperature. In both the path ways, the initial state and the final state are the same. In one pathway (Pattern 1), both fully α -helical polypeptides and fully β -structural polypeptides separately coexist at inadequate temperatures and the ratio of the two forms of polypeptides changes with the rise of temperature. The other pathway (Pattern 2) contains polypeptides each of which has α -helical moieties and β -structural moieties as intermediates at inadequate temperatures and the existing ratio of the two types of moieties changes in each polypeptide with the rise of temperature. Pattern 2 emphasizes that, at inadequate temperatures (two of the center in four pieces), there is neither the fully α -helical state drawn on the left edge nor the fully β -structural state drawn on the right edge. In both the cases, the inter-molecular β -structures are considered to be formed between polypeptides, since the intra-molecular formation of β -structures can be anticipated only in extremely dilute polypeptide solutions. Then, the inter-molecular aggregation is anticipated to occur, when the β -structural polypeptides begin to exist in the former pathway and when the ratio of the β -structural moieties increases to some degree in the latter pathway. The two-state model appears likely to assume equilibrium between the fully α -helical polypeptides (N) and fully β -structural polypeptides (D) such as Pattern 1 in **Fig. 13** for denaturations and structural changes of proteins. However, Pattern 1 in **Fig. 13** would be concluded to be unnatural, as Pattern 1 in **Fig. 11** is impractical. This is the same in the α -helix to coil transition or another type transition. It is unrealistic that only some protein molecules are

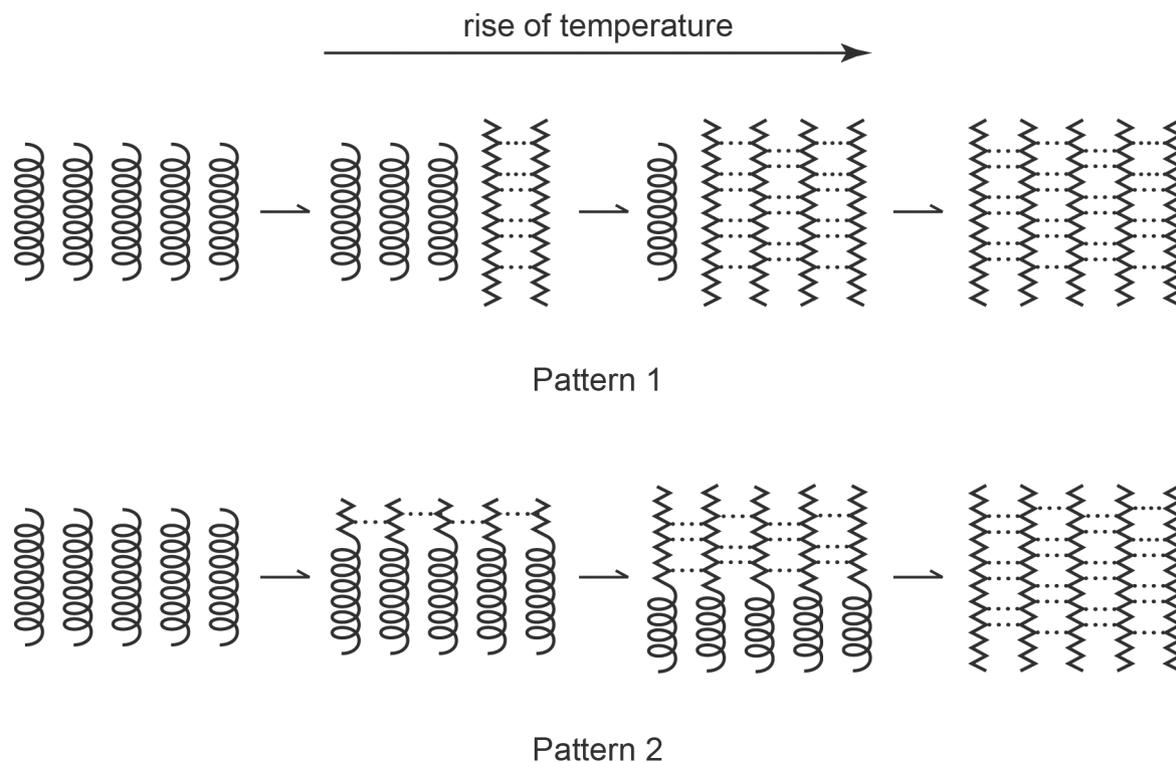


Fig. 13 Schematic drawing of two pathways of α -helix to β -structure transition with rise of temperature. Spiral rod: α -helical moiety, zigzag moiety: β -structural moiety, small dots between zigzag moieties: hydrogen bonds formed between polypeptides. This drawing emphasizes a difference of structural changes at inadequate temperatures to complete the transition (two of the center in four pieces of each). Pattern 1: fully α -helical polypeptides coexist with fully β -structural polypeptides and the amount of α -helical ones decreases with rise of temperature in an inadequate temperature range, Pattern 2: α -helical moieties of each polypeptide partially transforms to β -structural moieties and the amount of α -helical moieties decreases with rise of temperature in an inadequate temperature range.

influenced by a denaturing factor and the other molecules remained uninfluenced in the same solution. A denaturing factor is expected to influence every protein molecule equally, suggesting that Pattern 2 in Fig. 13 is more realistic than Pattern 1. It is important in Pattern 2 of Fig. 13 that there are neither the fully α -helical polypeptides (N) nor fully β -structural polypeptides (D) in an inadequate range of temperature. At a certain temperature in this temperature range, a protein adopts particular structures, which are neither N nor D defined in Scheme 3. The α -helix to coil transition of PLGA would occur with the rise of temperature in a wide temperature range in a similar manner to Pattern 2 in Fig. 13. In the case of PLGA, the transition occurs within each polypeptide without the inter-molecular interaction. Simply, the α -helical moiety decreases and the random coil moiety increases in each polypeptide with the rise of temperature. Important is that the denatured state of PLGA also variously changes depending on the temperature and that there are neither the fully α -helical polypeptides (N) nor fully random coil state polypeptides (D) in an inadequate temperature range.

Generally, when the intensity of the denaturing factor is altered, the denatured state of protein necessarily changes and is never fixed uniformly. Such a phenomenon cannot be explained under the assumption of equilibria as expressed by Scheme 3, which unmistakably indicates that the constant denatured D state coexists with the native N state under every condition. The present question cannot be solved by the three-state model, which assumes an intermediate species between N and D or other modified models of the two-state model. It is emphasized that the D state in these models changes depending on the pH, temperature, and concentrations of denaturants and any D state does not exist before the denaturation and the N state does not exist after the complete denaturation. It should be noted that there is neither fully native state (N) nor fully denatured state (D) under a half-finished denaturing condition.

One more problem is whether the denaturations or structural changes of proteins occur reversibly or not. In practice, it may be well recognized that the structural changes of proteins are not reversible under many condi-

tions, although, needless to say, ordinal chemical reaction equilibria are reversible as expressed by **Scheme 4**. The recoveries of structural changes of BSA, myoglobin, and α -lactalbumin have been observed upon cooling after the heat treatments in restricted temperature ranges (**Fig. 7**). However, a particular equilibrium relationship never exists between the N state and a constant D state, since the D state differs depending on temperature. This is the same for the thermal denaturations of homo-polypeptides (**Fig. 9**). The α -helix of PLGA is completely reformed upon cooling after the heat treatments in a wide temperature range, but the denatured state (the disrupted degree of the structure) differs depending on temperature (**Fig. 9(A)**). In the case of PLL, the α -helix is hardly reformed upon cooling and the denatured state sharply changes depending on temperature as seen in **Fig. 9(B)**. In each structural change of protein or homo-polypeptide, a certain D state may coexist with the N state only under a restricted condition which is not so different from the original condition. However, it is doubtful whether the relationship between the N state and D state is reversible even in such a hypothetical restricted condition. The case of thermal denaturation has been stated here. In the cases of denaturations by denaturants such as urea and guanidine, the examinations of recoveries of protein structures have been attempted only by dilutions of denaturants^{46, 50, 55}. In the case of surfactant denaturations, the examinations of structural recoveries require the addition of a third material³⁸⁻⁴², as mentioned above. Important is that the "recovery" here is different from the "reversibility of the chemical equilibrium". It is considered that the concept of the two-state model might be based on the observations that a certain D state partially or perfectly is returned to the N state when the denaturing condition is returned to the original condition. Indeed, the denatured structures of proteins are partially or perfectly recovered, when the denaturant concentrations are diluted, or the ascending temperatures are descended, or when the unfavorable values of pH are adjusted to appropriate ones. These observations might lead to a wrong concept that the relationship between N and D, as defined in **Scheme 3**, is reversible. Otherwise, many investigators have been obliged to assume the reversible equilibrium expressed by **Scheme 3** in order to interpret the denaturations of proteins as same as an ordinal reversible chemical reaction. It should be noted that the recoveries in the abovementioned observations are completely different from the reversibility in the equilibrium of an ordinal chemical reaction as expressed by **Scheme 4**, in which "every reaction species more or less exists" under every condition.

There would be no evidence to support the assumption that both the forward reaction and the backward reaction reversibly proceed in the denaturations or structural changes of proteins. It is rather reasonable that the original

structure of protein proceeds only in the forward direction to a particular structure determined by a given denaturing factor.

6 CONCLUSION

In conclusion, the binding of a certain amount of surfactant ions to proteins or homo-polypeptides transforms their native structures to particular structures without passing through intermediate structures, which might be induced by the bindings of fewer amounts of the surfactant ions. This mechanism is mainly based on the kinetic aspects of their surfactant-induced structural changes and the discontinuous nature of surfactant-protein complex formation observed in the electrophoresis measurements. On the other hand, it is irrational to use the two-state or three-state model for the denaturations or structural changes of proteins caused by denaturing factors, including surfactants. It is repeatedly emphasized that the two-state model is different from a general chemical reaction scheme such as **Scheme 4**, and modification of the two-state model does not solve this problem either. The process and the degree of denaturation or structural change of protein should be examined on the assumption that the denatured state differs, that is, the reaction differs with change of an optional denaturing factor. The present review reveals that the denaturations or structural changes of proteins are better studied using one-way reaction models with no backward processes rather than the reversible two-state reaction models or similar modified reaction models.

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