Critical Temperature of Secondary Structural Change of Myoglobin in Thermal Denaturation up to 130 °C and Effect of Sodium Dodecyl Sulfate on the Change

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Received: September 9, 2009; Revised Manuscript Received: December 17, 2009

The secondary structural change of horse heart myoglobin was examined in the thermal denaturation up to 130 °C. The original helicity of 82% gradually decreased to 67% with rise of temperature until 75 °C. Thereafter, it suddenly decreased to 24% at 90 °C and then slightly decreased to 14% at 130 °C. The helices of this protein were mostly destroyed between 75 and 100 °C. On the other hand, upon cooling to 25 °C from temperatures below 75 °C, the helicity completely recovered to the original value, but it did not after heating to temperatures above 80 °C. Thus, myoglobin maintains the reversibility of the structural change up to a temperature as high as 75 °C. This protein had another critical temperature around 90−100 °C in addition to 75 °C in the present thermal denaturation. Upon cooling to 25 °C after heating to temperatures above 80 °C, the extent of recovered helicity decreased with rise of temperature before cooling. The additive effect of sodium dodecyl sulfate (SDS) on the structural change of myoglobin differed below and above the critical temperature at 75 °C. In the temperature range below 75 °C where the structural change was reversible, the presence of SDS cooperated with the thermal denaturation to disrupt the structure. On the contrary, the presence of the surfactant more or less restrained the decrement of helicity at high temperatures above 85 °C. The helicity decreased and increased with an increase of SDS concentration upon cooling to 25 °C after heating to temperatures below 75 °C and after heating to temperatures above 85 °C, respectively. Then, upon cooling to 25 °C from any temperature, the helicity settled to a magnitude around 60% in the presence of the surfactant above 0.6 mM.

Introduction

Myoglobin is a well-known heme protein, which contains an abundant amount of helical structure. The structure of sperm whale myoglobin, which was a homologous protein of the present horse heart myoglobin, was adopted in the initial X-ray crystallographic study by Kendrew et al.[1,2] Furthermore, Edmundson[3] and Watson[4] assigned that the myoglobin has eight helical segments consisting of 118 amino acid residues out of a total of 153 residues. Thereafter, this protein has been investigated from various angles. However, the structural change of myoglobin in the thermal denaturation has not been investigated so much. The thermal denaturation of myoglobin has been investigated so far mainly by measurements of infrared spectroscopy or thermal analysis,[5−8] in which the protein concentration is about two orders higher than those adopted in usual spectroscopic studies. Although the circular dichroism (CD) method, which is probably most sensitive to secondary structure of protein, has been accompanied in some of these studies of myoglobin, a detailed secondary structural change of the protein has not been examined there.

On the other hand, the surfactant−protein interactions have extensively been investigated for more than a half century.[9−15] In the studies of the interactions, sodium dodecyl sulfate (SDS) has been most frequently adopted as a representative ionic surfactant. This surfactant and many other surfactants have been used as tools to isolate, solubilize, and manipulate many proteins for subsequent biophysical and biochemical characterization.[16] These applications skillfully depend on the unique nature of surfactant as an amphiphilic material. In these processes, however, the role and mechanism, which the surfactants play as tools, are not well-defined yet, in spite of many studies of the interactions between surfactants and proteins. In the progress of the studies, the structural change of myoglobin by surfactant has not been studied so much as compared with those of proteins such as serum albumins. When we referred "myoglobin-structural change-surfactant" to CAPLUS and MEDLINE in SciFinder Scholar, only four works appeared.[17−20] Above these, there are several related reports.[5−8,21−24] In the studies of the structural change of myoglobin by surfactant, changes of characteristic heme[25−27] and its environment have been examined variously, but changes of the secondary structure have been rather unnoticed.

The present study shows that the secondary structure of myoglobin is proof against the heat treatment. The helicity gradually decreases with rise of temperature until 75 °C, and it completely recovers upon cooling to 25 °C from temperatures below this temperature. This protein maintains the reversibility of the structural change up to temperatures as high as 75 °C. Although most of the helices are destroyed above 80 °C, the decrement of helicity by the heat treatment is restrained in the coexistence of SDS. More recently, the present authors have studied the protective effect of SDS on the structures of serum albumins in the thermal denaturation[15,28] and urea denaturation.[29,30] It should be noted that a similar effect of the surfactant has been observed on the structure of another protein, myoglobin, in the thermal denaturation at high temperatures.

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10.1021/jp908700j
Published on Web 01/29/2010

Materials and Methods

Myoglobin (horse heart) was purchased from Sigma Chemical Co. SDS[15,28−30] was obtained from Fluka Chemie AG. A sodium phosphate buffer of pH 7.0 and ionic strength 0.014[15,28−30] was exclusively used to prepare each solution. The CMC of SDS in the buffer was 5.6 mM at 25 °C.[31] The concentrations of myoglobin were determined spectrophotometrically using $\varepsilon_{409} = 1.71 \times 10^5\ M^{-1}\ cm^{-1}$.[32] The final protein concentration was adjusted to 10 μM.

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. Absorbance was measured with a Hitachi U-3310 spectrophotometer at 25 °C. In the CD measurements, we used a special high-temperature cell holder system ordered from Japan Spectroscopic Corporation to heat an aqueous solution to a temperature more than 100 °C.[15] Temperature was controlled under nitrogen gas of 1.0 MPa. We also confirmed by NMR measurement that a degradation of the dodecyl sulfate ion was not induced even at 130 °C.[15] A real temperature of the solution in the CD cell was determined with a thermocouple detector in each measurement. The CD spectrum was measured at a desired temperature after keeping the protein solution at the temperature for 30 min. When the temperature was raised or cooled to another temperature through the measurements or when the protein solution was kept at a certain temperature, the cell containing the protein solution was protected from the ultraviolet beam. This is because the irradiation of such an ultraviolet light disrupts the structure of protein.[33,34] The α-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.[35] The simulation was carried out in the wavelength region 200−240 nm at 1 nm intervals. Since myoglobin (sperm whale) is one of the reference proteins adopted in the determination of the reference spectra by Chen et al.,[35] the CD spectrum of the present protein can be well simulated by using their reference spectra.[17,18]

Results and Discussion

Thermal Effect on the Structure of Myoglobin. Myoglobin contains an abundant amount of helical structure. The helicity of 82%[17,18] has been estimated by the simulation of the CD spectrum at 25 °C, using the reference spectra of protein secondary structures determined by Chen et al.[35] Although the estimated helicity[17,18] is slightly higher than the values[1−4,35−38] determined so far for the native myoglobin, this magnitude is used as the original helicity of the protein throughout the present work. The CD spectra of the present myoglobin at several temperatures are presented in Figure 1. The CD spectrum, indicative of an α-helix, was mostly preserved up to 70 °C. However, the spectrum abruptly changed beyond 80 °C and lost the α-helical shape at 90 °C. The spectrum did not change so much beyond 90 °C. Figure 2 shows both the helicity changes of myoglobin with rise of temperature up to 130 °C and upon cooling to 25 °C from each temperature. The helicity gradually decreased with rise of temperature until 75 °C and thus reached 67% at this temperature. Thereafter, the helicity suddenly decreased to 24% with rise of temperature until 90 °C. The helicity more decreased to 14% beyond 110 °C. Compared with the fact that the helicity of bovine serum albumin (BSA) is sharply destroyed above 50 °C,[15,28] the structure of myoglobin apparently has a characteristic nature to stand heat. The present result indicates that the helices of myoglobin are rather stable until 75 °C, but they suddenly become unstable beyond this

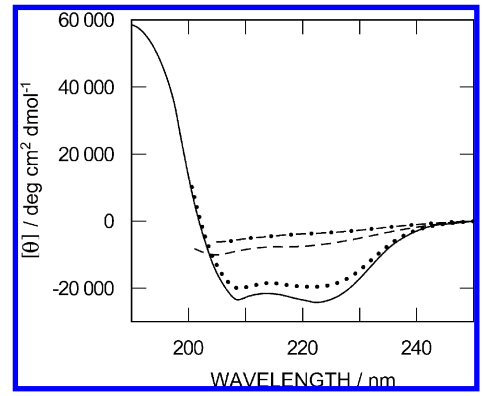

Figure 1. CD spectra of myoglobin at 25 (actual line), 70 (dotted line), 90 (broken line), and 130 °C (dot dashed line).

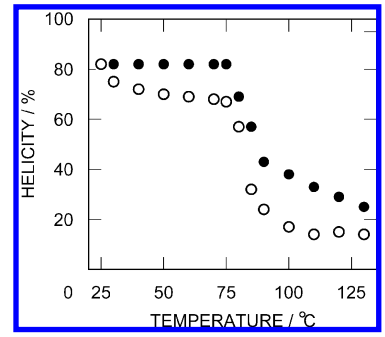

Figure 2. Change of myoglobin helicity in the thermal denaturation up to 130°C. ○, helicity upon keeping at each temperature of abscissa; ●, helicity upon cooling to 25°C from each temperature of abscissa.

temperature. Most of the helices of myoglobin were destroyed in a limited temperature range between 75 and 100 °C. Especially, a disruption of the helical structures was so great with rise of no more than 5−10 °C from 80 °C. The helicity of myoglobin has not been affected by the heating beyond 100 °C as seen in Figure 2, while that of BSA continues to decrease in this temperature range.[15]

Also upon cooling to 25 °C from high temperatures, the helicity of myoglobin showed a chracteristic feature. When the myoglobin solution was heated to temperatures below 75 °C and then cooled to 25 °C, the helicity completely recovered to the original magnitude, but it did not after heating to temperatures above 80 °C (Figure 2). The helical structures of myoglobin are perfectly reformed upon cooling to 25 °C from temperatures below 75 °C; that is, the helicity change is reversible in the thermal denaturation up to 75 °C, and it becomes irreversible beyond this temperature. It is noteworthy that the myoglobin maintains the reversibility of the structural change up to temperature as high as 75 °C. The thermal denaturation of myoglobin is considered to have a critical temperature around 75−80 °C. This critical temperature of myoglobin is apparently higher than the corresponding critical temperature, 50 °C, of BSA.[15,28] This also indicates that the structure of myoglobin is proof against the heat treatment.

Upon cooling to 25 °C after heating to temperatures above 80 °C, the recovered helicity decreased with rise of temperature before cooling. The structure of the protein is so damaged beyond 100 °C that the recovery of helical structure is further restrained. In the thermal denaturation, this protein might have another critical temperature around 100 °C in addition to 75−80 °C.

The above examination is for the secondary structural change of myoglobin. We also examined the tertiary structural change

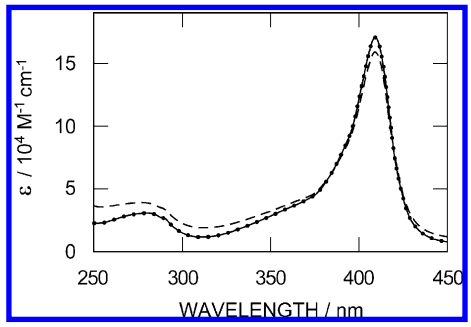

Figure 3. Absorption spectra of myoglobin at 25°C before heating (actual line) and upon cooling to 25°C after the heat treatments at 75 (dotted line) and 95°C (broken line).

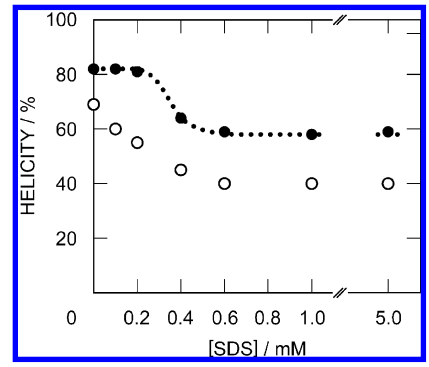

Figure 4. Dependence of myoglobin helicity on the SDS concentration upon keeping at 60 °C (○) and upon cooling to 25 °C from this temperature (●). The dotted line indicates the helicity change in the ordinal SDS denaturation at 25 °C.

and the heme environment change of the protein by the measurements of the absorption spectra in a wide wavelength region containing a Soret band.[25−27] The absorbance measurements were made only at 25 °C. The absorbance of the heated protein solution was measured after cooling to 25 °C. Figure 3 shows the absorption spectra of myoglobin at 25 °C before heating and upon cooling to 25 °C after the heat treatments at 75 and 95 °C. Upon cooling to 25 °C after the heat treatment at 75 °C, the whole spectrum of the protein was almost perfectly recovered to that with the original shape and intensity at 25 °C before heating. However, it was not recovered upon cooling to 25 °C after the heat treatment at 95 °C. These suggest that the tertiary structural change of the protein is also reversible until 75 °C according to the recovery of absorbance change due to aromatic amino acid residues. A similar reversibility can be anticipated for change of the heme environment in the temperature range below 75 °C. It is already known that the removal of the heme group from myoglobin unstabilizes the native conformation.[18,22,25−27] These suggest that both the heme and its circumstance are not so damaged in the thermal denaturations up to 75 °C. This might be deeply related to the aforementioned reversibility of the secondary structure in this temperature range. The present absorbance change suggests that the tertiary structure of myoglobin also loses the reversibility in the thermal denaturation beyond 75 °C. The aforementioned restraint of the helical structure recovery above 80 °C, especially beyond 100 °C, is considered to be caused by further changes of the tertiary structure and heme environment.

SDS Effect on the Structural Change in the Thermal Denaturation. The secondary structure of myoglobin is extremely susceptible to the SDS denaturation around room temperature.[17,18,24] The myoglobin−SDS system shows some features[17,18,24] which are not usually observed in other protein−surfactant systems.[13,17] The helicity of myoglobin decreases down to 58% below 0.6 mM SDS at 25 °C.[17,18] This SDS concentration region is much lower than the CMC in the present buffer. The molar absorption coefficient of myoglobin around the Soret region also drastically changes in similar low SDS concentrations, indicating an appreciable change of heme environment accompanying the secondary structural change of this protein.[17,18] Indeed, these SDS concentrations are one order lower than its concentrations where the conformations of other proteins[9−14,39,40] are affected; that is, the myoglobin is exceptionally susceptible to the SDS effect. Related to this, there is a particular suggestion that SDS interacts with myoglobin in a monomeric form, whereas a cationic surfactant does not,[23] although surfactant monomers are generally considered to interact with proteins, bringing about micelle-like aggregates.[41] There is one more characteristic in the interaction of SDS and myoglobin at room temperature: the binding of SDS proceeds with the surfactant concentration even after the secondary structural change is completed,[17] while both are generally saturated at the same surfactant concentration in the cases of other proteins.

Figure 4 shows the SDS effects on the helicities of myoglobin upon keeping at 60 °C and upon cooling to 25 °C after the heat treatment at 60 °C. Upon keeping at 60 °C, the helicity of myoglobin decreased with an increase of SDS concentration. The final helicity was 40% above 0.6 mM SDS. This tendency of the SDS effect was observed upon keeping at temperatures below 75 °C. In this temperature range, the addition of SDS of such low concentrations cooperates with the thermal denaturation to disrupt the helical structure; that is, the SDS denaturation simultaneously proceeds with the thermal denaturation. On the other hand, upon cooling to 25 °C after the heat treatment at 60 °C, the helicity was recovered to a level of SDS denaturation of each concentration at 25 °C before heating. The cooling treatment from temperatures below 75 °C appears likely to rearrange the state of the myoglobin−SDS complex similarly to that formed at each SDS concentration at 25 °C before heating. As mentioned above, upon cooling to 25 °C from temperatures below 75 °C in the absence of SDS, the helicity of myoglobin is reversibly recovered to the original magnitude. Therefore, myoglobin is considered to suffer the same denaturation effect of SDS as the protein in the native state without heating.

On the contrary, when the temperature was raised beyond 85 °C, the coexistence of SDS appreciably began to protect the helicity of the protein from the thermal effect; that is, the decrement of the helicity was restrained by the coexistence of the surfactant. Figure 5 shows the SDS effects on the helicities of myoglobin upon keeping at 90 °C and upon cooling to 25 °C after the heat treatment at 90 °C. Upon keeping at 90 °C, the protected helicity apparently increased with an increase in SDS concentration. The final helicity approached 40% at 5 mM SDS compared to 24% in the absence of the surfactant. A similar tendency was observed upon keeping at temperatures above 85 °C, but the final helicity at 5 mM SDS decreased with rise of temperature. Only upon cooling to 25 °C from 80 °C, the recovered helicity did not change apparently in the presence of SDS. This is probably because the helicity in this case is recovered by cooling just to a level attained by the SDS denaturation alone at 25 °C before heating. Upon cooling to 25 °C after the heat treatment at 90 °C (Figure 5), the recovery of helicity was accelerated in the presence of SDS less than 0.6 mM. The recovered helicity above 0.6 mM SDS was compatible with the helicity attained in the SDS denaturation of the corresponding concentrations at 25 °C before heating. Such a

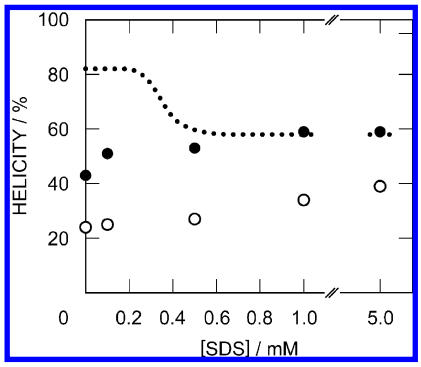

Figure 5. Dependence of myoglobin helicity on the SDS concentration upon keeping at 90 °C (○) and upon cooling to 25 °C from this temperature (●). The dotted line indicates the helicity change in the ordinal SDS denaturation at 25 °C.

reformation was observed upon cooling to 25 °C from temperatures between 85 and 130 °C. The same tendency has been observed in the effect of SDS on BSA structure upon cooling from high temperatures, and the extent of the reformation gradually reduces with rise of temperature before cooling.[15,28] The SDS effects on the myoglobin structure are observed in its low concentrations until 0.6 mM not only to decrease the helicity in the temperature range below 75 °C but also to recover the helicity upon cooling to 25 °C after the heat treatments above 85 °C. This might be correlated to the aforementioned fact that the helicity of myoglobin is accessible to the SDS denaturation in its low concentrations. On the other hand, only upon keeping at temperatures above 85 °C, higher SDS concentrations of millimolar order are required to protect the helicity. This is probably because the interaction of surfactant such as SDS with proteins is generally expected to become weak with rise of temperature. Nevertheless, the present result apparently indicates that the surfactant of low concentrations effectively interacts with myoglobin until temperature as high as 75 °C.

Comparison with BSA. We have recently examined the secondary structural changes of BSA at high temperatures up to 130 °C and the SDS effect on the changes.[15,28] When the present structural change of myoglobin is compared with the results of BSA, the former protein is clearly more stable than the latter in the thermal denaturation. Myoglobin loses the reversibility of secondary structural change beyond 75 °C as against 50 °C where BSA loses the reversibility. Interestingly, the protective effects of SDS on the structural changes are observed only at temperature ranges where both of the proteins lose the reversibility of the change. As a result, in the case of myoglobin with higher boundary temperature, the protective or recovering effect has been observed only upon keeping high temperatures above 85 °C and upon cooling to 25 °C only from this temperature range. Related to this, there is an empirical law in the denaturations of proteins by ionic surfactants such as SDS that the helical structure is partially formed in nonhelical proteins and proteins with lower helicity, whereas it is disrupted in proteins with higher helicity.[13,39,40] Myoglobin and BSA belong to the latter group. When both the proteins are heated at high temperatures where the reversibility of structures disappears, the presence of SDS acts to protect a part of the helical structures or to accelerate the reformation of the structure by the cooling. These seem to be due to a similar SDS effect to form helical structures in proteins with lower helicity in the empirical law. On the other hand, SDS necessarily interacts with myoglobin with high helicity to disrupt its helical structure to some degree in the temperature range below 75 °C.

The aforementioned difference and similarity between myoglobin and BSA have apparently been observed in the present work. We notice a well-known difference between myoglobin and BSA; that is, the myoglobin has no disulfide bridge, while BSA has as many as 17 disulfide bridges.[42] Most of the helical moieties of BSA are rather regularly linked by these disulfide bridges.[42] The helices formed in such moieties correspond to the helicity of 45% as against the original total helicity of 66%.[13,43] Although both the proteins consist of one polypeptide chain, the total construction of BSA with many disulfide bridges seems to be more structurally stable than that of myoglobin. Nevertheless, most of the helical structures of myoglobin are maintained up to 75 °C, and the structural change of this protein is reversible until 75 °C. The structure of myoglobin is distinctly more proof against the heat treatment at high temperatures compared with that of BSA. It is considered at present that the stability of myoglobin in the thermal denaturation might be due to the compactness of the protein molecule.[18,22,25−27] Compared to myoglobin, the BSA molecule does not have such a compactness. It has been found on the basis of the X-ray crystallographic study[44,45] that three domains of human serum albumin (HSA), which closely resembles BSA in both the primary structure and the steric structure, are separately distributed to form a large hollow. The three domains of the BSA molecule are also expected to be located in a manner similar to HSA. Therefore, the BSA molecule is anticipated to be less compact. The compactness of myoglobin is known to be mainly constructed by the hydrophobic interaction of the hydrophobic side chains of constituting amino acid residues.[22] This situation of myoglobin might induce the extreme instability in the SDS denaturation as well as in the denaturations by general denaturants, urea, and guanidine.[5]

Although there are such differences between myoglobin and BSA, it has become clear that the structures of both the proteins are protected or recovered by the presence of SDS under some particular conditions. These phenomena have been overlooked in many previous studies of interactions of these proteins with surfactants.

Conclusion

The secondary structural change of horse heart myoglobin was examined in the thermal denaturation up to 130 °C, and the SDS effect was also examined on the change. The following three facts are emphasized to conclude.

(1) The secondary structure of myoglobin was found to have a critical temperature around 75 °C. The change of the helical structure was reversible up to temperature as high as 75 °C; that is, the structure was partially disrupted with rise of temperature, and the lost structure was recovered by cooling. A similar reversibility was also observed in the changes of tertiary structure and heme environment of the protein in the same temperature range.

(2) The helices of this protein were mostly destroyed between 75 and 100 °C. Upon cooling to 25 °C from temperatures above 80 °C, the recovered helicity decreased with rise of temperature before cooling.

(3) The coexistence of SDS restrained the decrement of helicity by the heat treatment above 80 °C. This indicates that the secondary structure of myoglobin is protected to some degree by the interaction with the surfactant ions. It is noteworthy that such a protective effect of SDS has been observed on the structure of myoglobin as well as that of BSA[15] in the temperature range where both the proteins lose the structural reversibility.

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JP908700J