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UREA- AND THERMAL-INDUCED UNFOLDING OF BOVINE SERUM ALBUMIN

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In this work, the difference between pH-induced isomerization and urea-induced unfolding, and the urea- and thermal-induced unfolding processes in different isomeric forms of Bovine serum albumin (BSA) are studied experimentally. Our results show that urea-induced unfolding is a two-step, three-state transition in both the N-form and the B-form, but a single-step, two-state transition in both the E-form and the F-form. At the same time, thermal-induced unfolding shows a two-state transition in all isomeric forms, but the cooperativity of unfolding is different.

Keywords: Isomerization; pH; urea denaturation; thermal denaturation; multidomain protein; Bovine serum albumin.

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1. Introduction

Protein folding which describes an unstructured polypeptide chain folds to a stable and highly ordered tertiary structure with biological functions is a central problem in biochemistry and biophysics. Protein folding has been shown to proceed through different mechanisms.[1,2] The simplest mechanism involves a two-state transition which proceeds directly from the unfolded state to the folded state without any detectable intermediates. The unfolding–refolding processes by chemical denaturants under *in vitro* conditions have received great attention since various partially folded intermediates have been found to accumulate during equilibrium unfolding for a number of proteins.[3,4] However, the majority of previous experiments of protein folding–unfolding were carried out on several small or single domain proteins without disulfide bonds. Therefore, the folding–unfolding of large and multidomain proteins with disulfide bonds are of great interest.

For protein BSA (Bovine serum albumin), the pH-induced conformational changes have been studied by many methods.[5] There are four different conformational isomerization forms related to different pH, namely the normal form

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(N-form), the fast form (F-form), the extended form (E-form) and the basic form (B-form). Besides, urea-induced denaturation of BSA at neutral pH has also been widely studied.[6] During the urea denaturation at neutral pH, it has been shown that BSA follows a two-step and three-state transition, that is, there exists an intermediate state. Although the unfolding mechanism of N-form is reported, the unfolding mechanisms in other isomeric forms of BSA are still unclear. In this work, the difference between pH-induced isomerization and urea-induced unfolding for protein BSA is studied. The equilibrium unfolding processes in other isomeric forms are also studied, and the results are compared with behaviors of the N-form of BSA.

2. Materials and Methods

Pure (>98%) crystallized BSA from Fraction V was purchased from Roche Inc. (Germany) and used without further purification. Ultra pure urea was purchased from Nanjing Wanqing Corporation. All other reagents used in our experiment were of analytical grade. The BSA were freed from dimer passing through Sephacryl-S100(HR) gel filtration column.

Protein BSA was dissolved in 60 mM sodium phosphate buffer to make a stock solution with 2 μM concentration. The pH value of this solution was adjusted by HCl. Then the solution was incubated for 2–3 h at room temperature before further measurements. Stock solution of protein BSA was prepared in 60 mM sodium phosphate buffer at different pH values. The final protein concentration is 2 μM. In the denaturation experiment, different amounts of urea were added to the protein solutions to get the desired concentrations of denaturant. The final solution mixture was incubated for 10–12 h at room temperature before fluorescence measurements.

Tryptophan emission fluorescence measurements were carried out using a fluorescence spectrophotometer (Jasco FP-6500) with a cuvette of 1 cm light path. All fluorescence spectra were measured at protein concentration of 2 μM at $T = 25°$C. Excitation and emission slits were set at 3 and 5 nm, respectively. Intrinsic fluorescence was measured by exciting the protein solution at 280 nm and emission spectra were recorded in the wavelength range of 295–400 nm. All data were collected at room temperature. Urea-induced denaturation was monitored by the change in fluorescence intensities at 342 nm for the N-form, the B-form and the F-form, and at 329 nm for the E-form.

Steady-state far-UV CD measurements were made by using a Jasco-810 spectrometer with thermostating to within 0.1°C of the desired temperature. CD spectra were measured in the far-UV region under constant nitrogen flush by using a 1 cm path-length cell containing 0.2 μM protein in 4 mM sodium acetate buffer. The sample was heated by using water path with a heating rate of 1°C/min interfaced with the microcomputer. The sample was equilibrated for 3 min before measurement. Spectra were collected with a scan speed of 50 nm/min and with a response time of 1 s at a desired temperature. The results are expressed as mean residue ellipticity (MRE), and the alpha-helical content of BSA was calculated from the MRE value at 222 nm as described by Chen *et al.*[7]

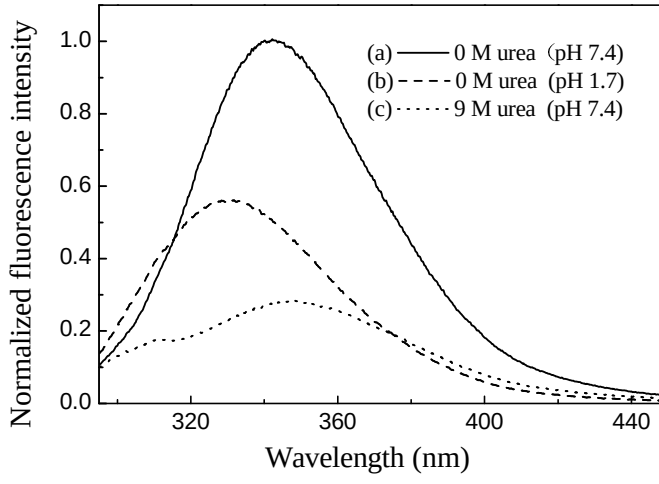

Fig. 1. Fluorescence emission spectra of 2 μM BSA after excitation at 280 nm. (a) Native state; (b) acid-induced isomerization form; (c) urea-induced unfolded state.

3. Results and Discussion

To understand the difference between the pH-induced isomerization and the urea-induced unfolding, we compare the tryptophan fluorescence emission spectra of BSA under neutral conditions with those obtained at external pH values and those in the urea-induced completely unfolded state (9 M). Figure 1 shows the emission spectra in the native state (with zero urea and pH = 7.4, curve (a)), in the acid-denatured state (with zero urea and pH = 1.7, curve (b)), and in the urea-denatured state (with 9 M urea and pH = 7.4, curve (c)) in which the protein is considered to be in the random coiled state. As can be seen from Fig. 1, the intensity of fluorescence emission decreases along with a blue shift with 13 nm of the positions of maximal emission from 342 to 329 nm as the pH decreases from neutral to extreme acid (curves (a) and (b)). This suggests that an acidification of BSA makes the tryptophan residue be in a more apolar environment. In general, when the tryptophan is settled in an apolar environment, its fluorescence emission intensity should increase. In contrast, the emission intensity observed in our experiment decreases. One possible reason is that, the effect of fluorescence quench becomes large since some specific quench groups, such as protonated carboxyl, protonated imidazole and tyrosinate anion, are close to the tryptophan after the decrease in pH.

The fluorescence spectrum of completely unfolded BSA in 9 M urea demonstrates that the position of maximal emission has a red shift from 342 to 348 nm along with a decrease in fluorescence intensity (curves (a) and (c)). This red shift indicates that more tryptophan residues of the protein are exposed to a polar environment. Such an exposure actually implies the unfolding. Moreover, we can see that there is a small peak at 310 nm in the fluorescence emission spectrum of urea-

induced denaturation state of BSA (curve (c)). The peak is attributed to tyrosine emission. However, such a peak cannot be observed in the native state, and also cannot be observed in the acid-induced isomerization state. These can be explained as that there exists fluorescence resonance energy transfer from tyrosine to tryptophan in the native state. Due to an increase in distance between tryptophan and tyrosine during urea-induced unfolding, the efficiency of fluorescence resonance energy transfer from tyrosine to tryptophan gradually decreases. Thus, a small emission peak of tyrosine can be observed. However, in the acid-induced isomerization, there is no emission peak of tyrosine since the distance between tryptophan and tyrosine is small and the efficiency of fluorescence resonance energy transfer is large. This implies that the local conformation of tryptophan becomes compact, which is consistent with the blue shift of the position of the maximal emission.

The mechanisms of pH-induced isomerization and urea-induced unfolding of protein have been widely studied experimentally and theoretically.[8,9] Many studies showed that the mechanism of pH-induced isomerization is related to an increase of electrostatic repulsion between like charges in protein which results in a large tendency of isomerization. For the urea-induced unfolding, Daggett *et al.* carried out molecular dynamics simulations for protein chymotrypsin inhibitor 2 (CI2), and demonstrated that urea denatures protein CI2 via both direct and indirect mechanisms. That is, urea interacts directly with the polar residues and the peptide backbones. As a result, non-native conformations become stable. Further, urea can alter water structure and dynamics, thereby diminishing the hydrophobic effect and enhancing solvation of hydrophobic groups. Thus, it is clear that urea-induced unfolding, mainly, is attributed to the changes of hydrophobic interactions, and pH-induced isomerization is mainly attributed to the electrostatic interactions. Our results show that the conformational changes during urea-induced unfolding are completely different from those during pH-induced isomerization. These, really, are related to the mechanisms of denaturation mentioned earlier.

To understand the difference of unfolding of different isomeric forms of BSA, urea-induced unfolding processes in different pH isomerization forms were studied. Figure 2 shows the urea-induced denaturation curves in the N-, the F-, the E- and the B-form, respectively. For the N-form, the decrease of tryptophan fluorescence intensity at 342 nm, shows a complex manner when the concentration of urea increases. The loss of tryptophan fluorescence emission intensity occurs in two steps. Initially, there is a rapid decrease in tryptophan fluorescence in the region of low concentration of urea. The fluorescence intensity decreases by 25% when the urea concentration is about 2 M. However, when the urea concentration is between 2 and 3 M, there is no change in tryptophan fluorescence intensity, that is, there is a plateau, indicating the formation of an intermediate in this region. Beyond 3 M urea, fluorescence intensity decreases gradually as the urea concentration increases and the second transition ends at 8 M urea. These results demonstrate that urea-induced unfolding of BSA at neutral conditions follows a two-step and three-state

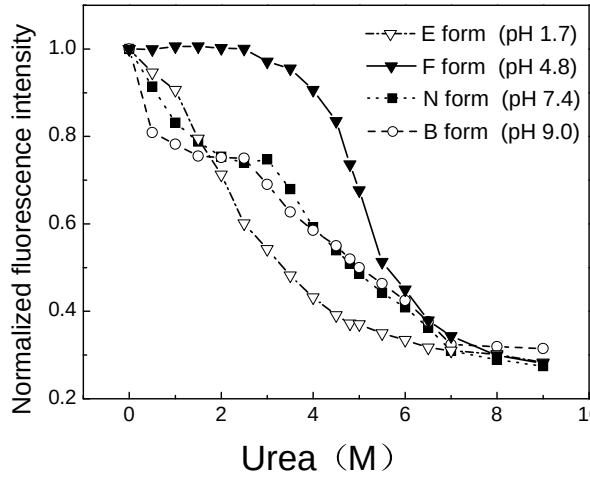

Fig. 2. Urea-induced unfolding of BSA in different isomerization forms monitored by tryptophan emission fluorescence.

transition with the accumulation of an intermediate between the native and the completely unfolded states. This is in agreement with the previous observations made for albumin.[10]

For the F-form, below 2.5 M urea, the fluorescence intensity at 342 nm remains unchanged (Fig. 2), indicating an increase of the protein resistance to chemical denaturation when pH is changed from neutral to slight acid. When the urea concentration is beyond 2.5 M, there is a single sharp transition between the initial and final states, indicating that urea-induced unfolding in the E-form is cooperative and the transition curve is sigmoidal. This shows that urea-induced unfolding at slightly acid conditions is a single-step, two-state transition, without detectable equilibrium intermediate. The transition was also observed in human serum albumin.[11] Since Khan *et al.* have demonstrated that the transition from the N-form to the F-form involves unfolding and separation of domain-III from the rest of the molecule,[12] the above result presents a direct evidence that the formation of the intermediate in the unfolding transition involves unfolding of domain-III. Furthermore, Fig. 2 demonstrates the equilibrium denaturation curve of BSA in the B-form. As can be seen from the curve, that there also exists an intermediate, around 1.5–2.5 M urea concentrations, in the B-form lower than those in the N-form, suggesting that the stability of domain-III decreases when the pH values were changed from 7 to 9. The results also show that urea-induced unfolding in the B-form is also a two-step, three-state transition.

Figure 3(a) shows the far-UV CD spectra in 200–250 nm range under different pH-induced isomerization forms of BSA. The curve for the F-form has two minima, one at 208 nm and the other at 222 nm, characterizing the α-helix structure (Fig. 3(a)). BSA in the F-form contains around 57% of α-helix structure as

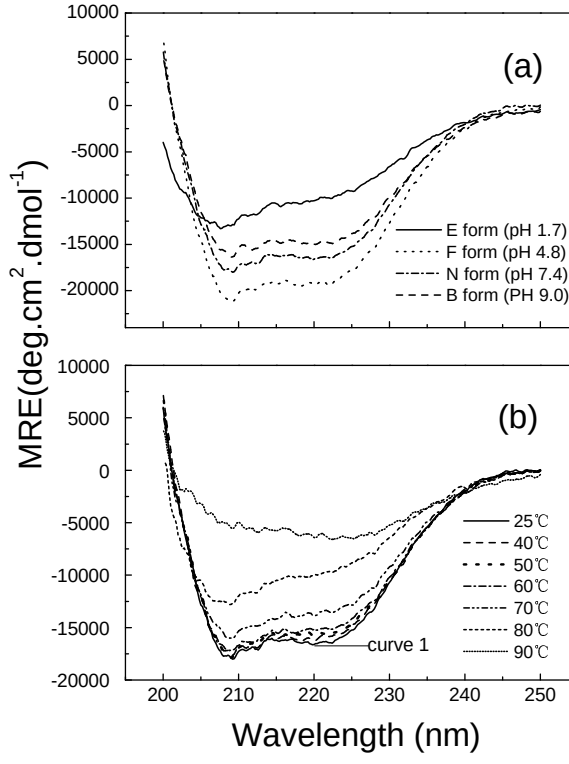

Fig. 3. Far UV CD spectra for different isomerization forms of (a) BSA and (b) thermal denaturation of native BSA in the N-form (0.2 μM) from 25°C to 90°C.

determined from the ellipticity value at 222 nm. At the same time, the helix contents calculated from MRE values at 222 nm, for the N-form and the B-form are found to be 47% and 41%, respectively. It shows that the helix content in slightly acid medium is larger than those in neutral and slightly alkaline medium. Furthermore, as can be seen from Fig. 3(a) a significant amount of α-helix structure is retained by the protein in the E-form which has been shown in an earlier study.[13] However, relating to the F-form, an approximate 32% decrease in MRE_{222} can be observed in the E-form.

The changes of the secondary structures of BSA during thermal unfolding in the N-form are monitored by using far-UV CD as shown in Fig. 3(b). Curve 1 shows the far-UV CD spectrum for BSA in aqueous solution at $T = 25$°C. The spectrum was characterized by the presence of minima at 208 and 222 nm which are the features of helical secondary structure. Our results are in good agreement with those by Itri *et al.*[14] As can be seen from Fig. 3(b), the helicity is decreased because of the decrease in ellipticity at 222 and 208 nm as the temperature increases. However, until $T = 90$°C, there still exists a large number of α-helixes. This indicates that

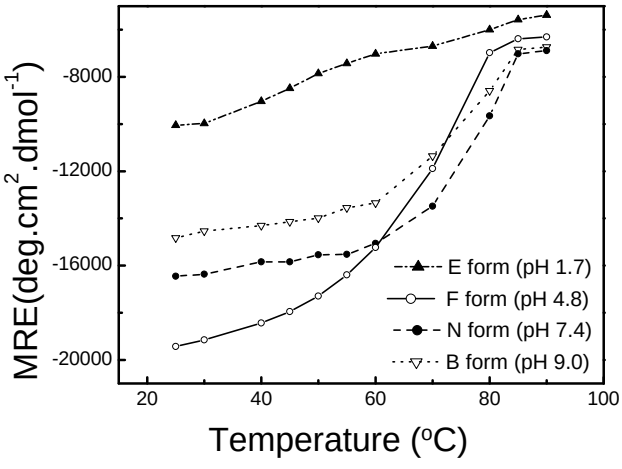

Fig. 4. Thermal denaturation of BSA monitored by far-UV CD at 222 nm for different isomerization forms.

BSA possibly does not completely lose its secondary structure even at $T = 90°C$ due to the constraint of disulfide bonds.

Figure 4 shows the temperature-induced unfolding of BSA in different isomerization forms. In the F-form, the transition was cooperative with no accumulation of the intermediate state. This is the typical feature of a two-state model. Similarly, the thermal-induced unfolding in the N- and the B-form also shows a two-state transition, but the thermal unfolding transition in the N- and the B-form, as suggested by the CD spectra, is less cooperative than that in the F-form. However, thermal unfolding transition in the E-form is basically a non-cooperative process.

These results demonstrate that the thermal-induced unfolding cooperativity for different isomeric forms is different. This clearly indicates that pH can influence unfolding cooperativity of BSA. Previously, Scheraga *et al.* showed a substantial change in the charge distribution for each type of amino acid residue with the change in pH.[15] These changes in the charge distribution may lead to the loss of favorable local electrostatic interactions, which appear to have a significant effect on the cooperativity of protein unfolding.

4. Conclusion

A comparison between pH-induced isomerization and urea-induced unfolding of protein BSA is carried out by observing the intrinsic fluorescence spectra. Our comparative analysis demonstrates that the structural changes of protein BSA during pH-induced isomerization are completely different from those during urea-induced unfolding, which mainly originates from their different mechanisms. Beside, we have provided clear evidence that pH not only alter urea-induced unfolding pathway of protein BSA, but also influence the thermal-induced unfolding cooperativity,

indicating that considering the changes of electrostatic interactions with pH is very necessary for exploring the folding mechanism of multidomain proteins.

Acknowledgment

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