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Influence of metal ions on folding pathway and conformational stability of bovine serum albumin

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Abstract

In order to understand the effects of metal ions on the structural stability of proteins, urea-induced unfolding transition of bovine serum albumin (BSA) in the presence of different kinds of metal ions (Zn^{2+}, Ca^{2+}, Cd^{2+} and Co^{2+}) are characterized by far-UV circular dichroism (CD) and fluorescence spectra. Under neutral condition, there is a significant deviation of the unfolding transition curves of free protein by fluorescence and CD spectra. However, the unfolding curves of fluorescence and CD spectra turn to be coincident in the presence of Ca^{2+} or Cd^{2+} ions, indicating that the tertiary and secondary structures of BSA unfold simultaneously, i.e. a cooperative two-state behavior, while the deviation of them is enhanced in the presence of Zn^{2+} or Co^{2+} ions, suggesting that the unfolding remains a three-state behavior. Our results provide evidence that the metal ions alter the urea-induced unfolding pathway of BSA. Furthermore, the change of free energy (ΔG), the midpoint of chemical denaturation (C_m) and the solvent-exposed surface (m) during the urea-induced unfolding process have also been obtained to characterize the effects of metal ions on the conformational stability of BSA.

Keywords: Bovine serum albumin; Denaturation; Fluorescence; Circular dichroism

1. Introduction

Protein folding is a hot topic of modern biophysics and biochemistry, and many aspects have been studied based on both theoretical and experimental approaches. It is well known that folding of some small proteins presents highly cooperative two-state behavior, while folding of multi-domain proteins with populations of partially folded states involves multi-state process or the intermediates [1]. The protein stability and folding pathways are closely dependent on the solvent milieu, e.g. various ionic compositions [2]. Salts are known to affect these in a variety of ways, such as specific and non-specific binding of ions to the protein molecules, electrostatic shielding of charges, etc. [3]. The interactions between ions and residues in proteins are important for protein stability, and a rational optimization of such interactions could enhance the stability of some proteins [2,4]. This actually suggests that specific binding of ions with residues in a protein has important implications for biological functions of proteins and also for the design of proteins with high thermo-stability by construction of ion-binding sites in proteins.

Albumin, the most abundant protein in mammalian blood plasma, involves in binding, transport and delivery of a range of endogenous and extrinsic small molecules or ions, such as fatty acids and metal ions [5]. Bovine serum albumin (BSA), which is homological with the serum albumin of human and other mammalian, has been intensively studied. It is a single polypeptide chain with 583 residues and consists of three domains (I, II, III), as shown in Fig. 1. Each domain contains two sub-domains. BSA shows a peculiar residue distribution, compared to that of typical proteins, with a high content of the charged residues (residues Glu and Lys). Therefore, serum albumin has strong interactions with anionic and cationic ligands [6,7], such as metal ions.

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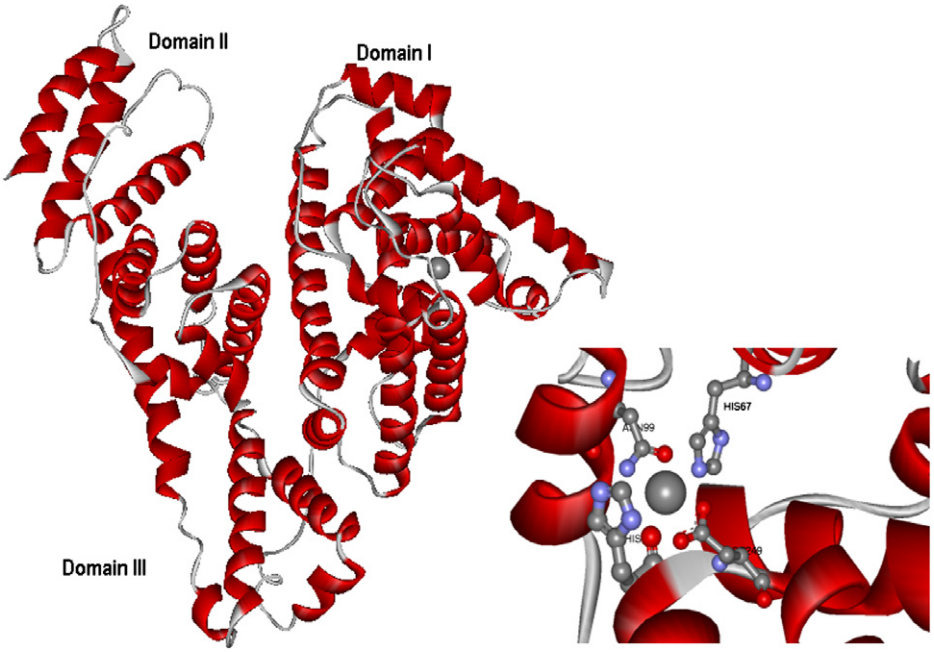

Fig. 1. The native structure of albumin and the coordinating residues with metal ions.

Many studies have been carried out on the chemical and thermal denaturation of BSA [8]. Although its domains are stabilized by various inter-domain forces, including hydrophobic, salt bridge interactions and natural borders (helical extensions) between different domains, the loss of conformation in three domains (I, II, III) is not simultaneous. The most labile part is domain III, while domains II and I are relatively stable [9]. Furthermore, anion-induced stabilization of albumin against urea denaturation is accompanied by the construction of ions-binding sites on the surface of domain III [10]. However, whether cations have similar effects on the stabilization of BSA against denaturants has been rarely reported, although the binding sites of metal ions in albumin have been extensively investigated [5,11]. In the present work, effects of some high affinity divalent metal ions (Zn^{2+}, Cd^{2+}, Ca^{2+}, Co^{2+}) on the folding pathway and conformational stability of BSA have been studied based on fluorescence and CD spectra. It is found that different metal ions behave different effects on the conformational stability under urea denaturation. In the presence of Ca^{2+} or Cd^{2+} ions, the unfolding of BSA shows a cooperative two-state process with the tertiary and secondary structures destroyed simultaneously, while in the presence of Zn^{2+} or Co^{2+} ions, the unfolding of BSA is of three-state.

2. Experimental

2.1. Materials

Pure (>98%) crystallized BSA from Fraction V was purchased from Roche Inc. (Germany) and used without further purified. Urea was purchased from Nanjing Wanqing Corporation. All other reagents ($ZnCl_2$, $CdCl_2$, $CaCl_2$, $CoCl_2$, EDTA and Tris–HCl) were of analytical grade without further purified. Solutions were prepared with Milli-Q super purified water. Concentrations of protein solution were determined spectrophotometrically as described in Ref. [12]. Concentrations of metal ions were determined by the titration of EDTA. The effect of Cl^- ion can be eliminated with buffer of Tris–HCl [10].

2.2. Fluorescence and circular dichroism spectra measurements

The intrinsic fluorescence measurements were performed on fluorescence spectrophotometer (Jasco FP-6500) with a cuvette of 1 cm light path. The excitation wavelength of intrinsic spectra was 280 nm and the emission spectra were recorded in the wavelength range of 295–400 nm. The bandwidths of excitation and emission slits were set as 3 and 5 nm, respectively. CD was recorded on a Jasco-810 spectrometer. The steady-state far-UV CD spectra of protein were recorded from 190 to 250 nm using a 1 cm path-length cuvette under constant nitrogen flush. Each spectrum was averaged by 5 scans. The α-helical content of BSA was calculated from the ellipticity value at 208 nm using the following equation [13]

$$\alpha\%\ \text{helix} = [(-[\theta]_{208} - 4000)/(33000 - 4000)] \times 100. \quad (1)$$

2.3. Chemical denaturation experiments

Solutions for experiments were prepared from stock solutions of protein and salts ($ZnCl_2$, $CdCl_2$, $CaCl_2$, $CoCl_2$) prepared in Tris–HCl buffer (pH 7.4). The concentration of

protein and salt were 2 μM and 1 mM, respectively. The complex solutions were incubated for enough time (>6 h) at 25 °C. On the chemical denaturation experiments, to a stock protein solution, different volumes of urea solution (10 M) were added to obtain the desired concentration of denaturant. The fluorescence and CD spectra of BSA were measured at 25 °C.

3. Results and discussion

3.1. Urea-induced unfolding of BSA

As an indicator of structural changes, the intrinsic fluorescence and far-UV CD spectra of BSA with metal ions are monitored on the urea-induced denaturation. The intensity of fluorescence at wavelength of 340 nm and ellipticity at 222 nm varying with the concentration of urea are shown in Figs. 2(a) and (c). The emission spectrum is sensitive to the tertiary environment of the fluorophores in a protein, while the ellipticity at 222 nm as a standard measure of helical content in a protein is used to estimate the secondary structural change of the protein [14–16]. At low concentration of urea, the fluorescence intensity and the value of ellipticity are little changed in a high level, and the BSA molecules remain in the native state although they are coordinated with the metal ions. However, there is a large decrease of the fluorescence intensity of BSA with Co^{2+} ions, while minimal increments of the fluorescence intensity of BSA with Zn^{2+}, Ca^{2+} and Cd^{2+} ions are present. The increments of the fluorescence intensity result from the increased surrounding polarity around aromatic residues due to the coordination bonds formed between metal ions and charged residues [16]. Meanwhile, there is somewhat change for CD spectra. Free BSA contains major amounts of α-helix (47%), random coil and a little beta sheet. For the cases of Zn^{2+} and Ca^{2+} ions, the content of helix decreases to 41% and 45%, respectively, and for the cases of Co^{2+} and Cd^{2+} ions, it increases to 53% and 50.5%, respectively. Although some strong binding sites of Co^{2+} or Cd^{2+} ions make the helical extensions strengthened at native state of BSA, these interactions are unstable in the presence of urea because there is a slow decrease for the cases of Co^{2+} or Cd^{2+} ions. Once the concentration of urea is beyond 4 M, there is a sharp decrease of both the fluorescence intensity and ellipticity, suggesting that the unfolding process follows the sigmoidal curves. With higher concentration of urea, both the fluorescence intensity and ellipticity become unchanged again, and the differences of the fluorescence intensity and ellipticity between the cases with and without metal ions disappear basically. This means that the unfolded states are independent of the existence of the metal ions presumably due to the lost of the coordination bonds. Even though, there remain about 10% of helices as indicated by the non-zero value of the ellipticity at 8 M urea.

Interestingly, the fluorescence intensity of BSA with Co^{2+} ions decreases largely, especially in the native state, compared with the case without metal ions. This is because that the addition of Co^{2+} ions results in the fluorescence quenching owing to the complicated coordinate interactions between Co^{2+} and the BSA molecule [14,17]. In order to understand the origin of the decrease of the fluorescence intensity, the changes in the UV-absorption spectrum of BSA with and without Co^{2+} ions are measured (data not shown). It is found that there is a strong absorption in the ultraviolet region of 280 nm for Co^{2+} ions. This is related to the special feature of Co^{2+} ions. Paramagnetic Co gives rise to the un-irradiation energy loss due to the intersystem crossing between the single state and triplet state for metal complex. There are three strong binding sites in BSA for Co^{2+} ions, and each has more coordinate bonds between the Co^{2+} ions and the residues. Among them, the binding of Co^{2+} ions with two to three imidazole nitrogen atoms of residues His105, His146 and His247 and one to two carboxyl oxygen atoms of residues Asp249, Asp107 or Asp108 is located in the inter-domains I and II [15–18], which is close to residues Trp214, Tyr148 and Tyr150, inducing a fluorescence energy transfer between the Co^{2+} ions and aromatic residues [17]. Therefore, the energy transfer between BSA molecules and the Co^{2+} ions can brings the decrease of fluorescence emission of BSA [14].

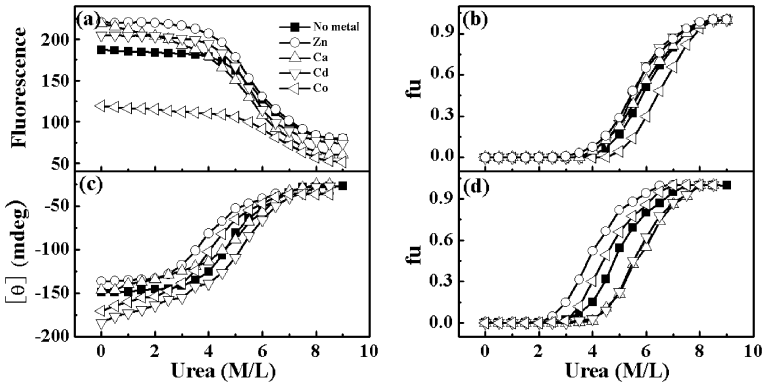

Fig. 2. The urea-induced equilibrium unfolding transition of BSA with and without metal ions (N, the solid squares, stands for no metal ions). The fluorescence intensity and the unfolding fraction measured at 340 nm (a and b); the ellipticity and the unfolding fraction measured at 222 nm (c and d).

From the data of fluorescence and CD spectra, the fraction of unfolded BSA molecules can be estimated using the following equation:

$$f_u = (y_n - y)/(y_n - y_u). \quad (2)$$

Here f_u is the fraction of unfolded molecule, y_n and y_u are the values of fluorescence intensity (or ellipticity) at the folded and unfolded states extrapolated from the pre- and post-transition baselines to the urea concentration under consideration [19–21]. The unfolding transitions of BSA are shown in Figs. 2(b) and (d). Comparing with the case without metal ions, the unfolding transition curve of BSA with Co^{2+} ions from the fluorescence intensity shifts to higher concentration of urea, indicating that Co^{2+} ions enhance the stability of the tertiary structure of BSA in urea-induced denaturation, while those with Zn^{2+}, Ca^{2+} and Cd^{2+} ions shift to lower concentration of urea, implying that the unfolding of the tertiary structure of BSA is accelerated. As shown in Figs. 2(d), the unfolding transition curves of BSA with Ca^{2+} and Cd^{2+} ions shift to higher concentration of urea comparing with the case without metal ions, implying that the helix stability of BSA is enhanced. In contrast, the helix stability is weakened in the cases of Zn^{2+} or Co^{2+} ions.

3.2. Comparison of the unfolding transitions of the secondary and tertiary structures

A study by Tayyab [10] has shown that anion-induced stabilization of human serum albumin prevents the formation of intermediate state during urea denaturation process due to the disappearance of the mid-step of unfolding transition curves measured by fluorescence spectra. Therefore, the unfolding transition of BSA becomes a two-state process (N ↔ D, corresponding to the native state to the denatured state) because of the stabilization of Cl^- on the domain III. The elucidation of the role of intermediate states is a major focus in the study of protein folding pathway. In order to investigate whether the urea-induced unfolding of BSA in the presence of Cl^- is a two-state or three-state transition, we compare the unfolding transition curves of fluorescence and ellipticity of BSA on the urea-induced unfolding process in Fig. 3. It is clearly seen that there is a significant deviation from the unfolding transition curves of the tertiary and secondary structures, indicating the existence of the partially unfolded equilibrium states during the unfolding process. Thus, the unfolding process of BSA in the neutral conditions remains of three-state (N ↔ I ↔ D, corresponding to the native state to intermediate state, denatured state). Comparatively speaking, a single probe is often not sufficient to characterize the folding pathway of a multi-domain protein. It is desirable to use multiple probes for characterization of the unfolding transition of BSA as it could more insight into the folding pathway of proteins. Fig. 3 also shows that the destruction of the secondary structure of BSA occurs earlier than that of the tertiary structure in the unfolding course, which owns to the native structure of BSA. BSA contains major amounts of α-helix, with two tryptophan residues (Trp135 and Trp214) embedded in different domains. Trp-135 (in domain I) is buried in a hydrophobic pocket of domain I, and Trp-214 (in domain II) is located in the interior of domain II [15]. It is known that the emission spectra of intrinsic fluorescence of a protein are related to the micro-environment of tryptophan in solution. Among three domains of BSA, the structural stability of domain III is weaker than those of domains II and I. Although domain III is stabilized by anions, its unfolding is easier and earlier than other two domains. Thus, the initial unfolding of BSA involves in the loss of helixes in domain III, while the structures of domain I and domain II are not changed apparently. As a result, the environments of residues Trp135 and Trp214 in domain I and domain II are not influenced in the early stage of unfolding, providing that the fluorescence intensity is at a low level compared with the ellipticity at the same concentration of urea.

The folding pathway of proteins is closely dependent on the solvent milieu. In order to obtain the effects of metal ions on the folding pathway of BSA, the unfolding transition curves of fluorescence and ellipticity spectra of BSA with metal ions on the urea-induced unfolding process are compared in Fig. 4. The unfolding curves of fluorescence and ellipticity spectra coincide together in the cases

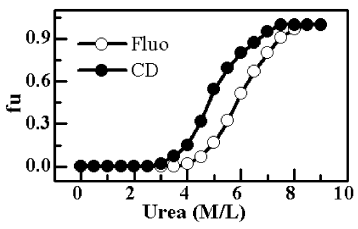

Fig. 3. A comparison of the unfolding fractions of BSA in the absence of metal ions based on the fluorescence and ellipticity spectra. The urea-induced unfolding curves of BSA measured by fluorescence and ellipticity at 222 nm of BSA are converted to unfolding fraction using Eq. (2).

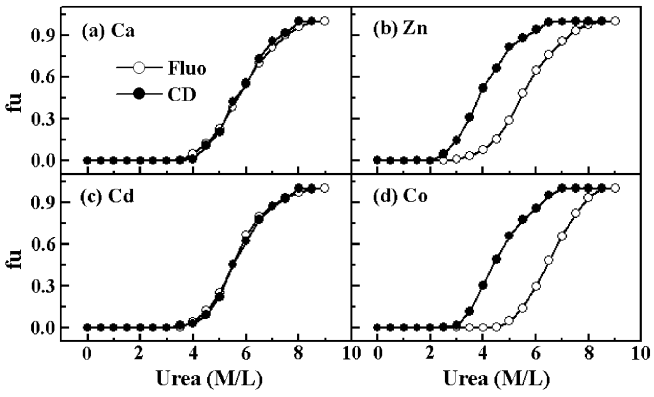

Fig. 4. The comparison of unfolding fractions of BSA in the presence of different metal ions based on the fluorescence and ellipticity spectra.

of Ca^{2+} and Cd^{2+} ions, suggesting that the unfolding of secondary and tertiary structure for BSA unfolds simultaneously as a cooperative two-state mechanism. Indeed, the unfolding transition presents a better cooperative behavior for the cases of Cd^{2+} ions. In contrast, the unfolding curves of fluorescence and ellipticity spectra in the cases of Zn^{2+} and Co^{2+} ions deviate far from each other, indicating the unfolding of secondary and tertiary structure for BSA are stepwise and the partially unfolded equilibrium states are presence in the unfolding process. The unfolding process of BSA with Zn^{2+} or Co^{2+} ions is of three-state behavior. Especially, the intermediate in the cases of Co^{2+} ions is obviously trapped by non-native interactions. On the basis of the results, the presence of metal ions affects the equilibrium unfolding pathway of BSA.

The change of folding pathway in the presence of metal ions as shown in Fig. 4 is related to the feature of binding ions. BSA involves in binding to a variety of metal ions with different specificities. There are strong affinities of Zn^{2+}, Ca^{2+}, Cd^{2+} and Co^{2+} ions with BSA molecules, especially for Zn^{2+} ions, which can substitute binding sites of other metal ions [11,22–26]. A tetrahedral Zn^{2+} site involves rich His residues, such as His67 and His247 are situated in the end of helixes of domain I and II [22]. Zn and high-spin Co show some similarities, including identical charges and similar radii, but the affinity ability and coordination number between them and BSA are different [23]. There are three strong binding sites in BSA for Co^{2+} ions, and each has more coordinate bonds between the Co^{2+} ions and the residues [24]. Ca^{2+} ions bind mainly to oxygen of carbonyl groups of residues in proteins. There are more binding sites at some loop regions between disulfide bridges in domain III with conserved carboxylate residues [5,26]. Cd^{2+} ions has been used as a probe for other metal ions in particular Zn^{2+} and Ca^{2+} ions in proteins [5,11]. The binding sites for Cd^{2+} ions are situated at the region of rich His residues and oxygen ligands of Asp and Glu residues. Although some binding sites of Cd^{2+} ions is competed by Zn^{2+} ions, Zn^{2+} ion binding occurs mainly through C=O group in protein, which can result in the conformational transition from helix to sheet or turn species, while Cd^{2+} ions as smaller ligands bind through C—N group in proteins [5–7,22]. Cd^{2+} is often as a good isomorphous replacement for Ca^{2+} ions in Ca-binding proteins due to the similar radii [5].

On the basis of the characterization of metal ligands, the influences of metal ions on the conformational stability of BSA are different. From the results in Fig. 2(c), it is clear that Co^{2+} ions enhances the stability of the tertiary structure of BSA in urea-induced denaturation, while Zn^{2+}, Ca^{2+} and Cd^{2+} ions decrease the stability of the tertiary structure of BSA. The binding of Co^{2+} ions with BSA is related to not only the directly participant residues but also those nearby residues. The increment of intra-molecular interactions between residues will strengthen the structural stability [17,24]. However, strong binding of Zn^{2+} ions in the protein causes an incompact structure due to the allosteric effect, which also makes Zn^{2+} ions replacing other metal ions [22]. Cd^{2+} ion also has high affinity with BSA. Although this smaller ligand does not interfere with the structure too much, the disruption of strong coordination interaction will affect the stability of BSA on the urea-induced denaturation [5,6]. For Ca^{2+} ions, there are many weak-binding sites in the surface residues (Asp or Glu) of BSA, which less influences the stability of the tertiary structure [5]. As for the change of the secondary structure of BSA in Fig. 2(d), Ca^{2+} and Cd^{2+} ions increase the stability of the helix in the unfolding process, while Zn^{2+} and Co^{2+} ions decrease the stability of the helix. The binding of Zn^{2+} ions in BSA will reduce the helix structure due to the destruction of the hydrogen bond in helix [11,22]. Co^{2+} ion binding in BSA has hysteretic effect, the first binding site at the helixes of domain I will drag other connected helixes to make more binding sites appear. In spite of the increment of intra-molecular interactions, it is unfavorable to the helix stability [17]. Ca^{2+} ions situated at some loops between helixes will stabilize the helix structure on the urea denaturation. For Cd^{2+} ions, the shielding of carboxylate ligands in a distant site indirectly of the coordination interactions is prone to the stability of secondary structure against urea [5,6]. Thus, for the cases of Ca^{2+} and Cd^{2+} ions the acceleration of the tertiary structure unfolding and delay of the second structure unfolding with respect to the case of BSA without metal ions lead to the coincidence of unfolding curves in Fig. 4. The unfolding transition curves of the tertiary and secondary structure are deviated for the cases of Zn^{2+} and Co^{2+} ions since the destruction of the secondary structures occurs earlier than that of the tertiary structure of BSA.

Considering the free energy change (ΔG) is directly related to the structural stability of proteins, we calculate it using the following equations [20,27–29]:

$$k_{eq} = f_u/(1 - f_u), \tag{3}$$

$$\Delta G = -RT \ln(k_{eq}). \tag{4}$$

Here k_{eq} is the equilibrium constant of unfolding reaction. The free energy ΔG, as a linear function of urea concentration, can be fitted to the Eq. (5) using a least-squares analysis,

$$\Delta G = \Delta G_{H_2O} - m\,[\text{urea}]. \tag{5}$$

Here ΔG_{H_2O} is the free energy change in the absence of denaturant, and m is the dependence of ΔG on urea concentration. As shown in Fig. 5, the ΔG curves of BSA with Zn^{2+}, Ca^{2+} and Cd^{2+} ions are lower than that without metal ions, while that with Co^{2+} ions is highest. These results indicate Co^{2+} ions strengthen the stability of tertiary structure for BSA. The changes of free energy in the absence of urea (ΔG_{H_2O}), the concentration of urea at half denaturation (C_m) and solvent-exposed surface (m) of BSA during the urea-induced unfolding process can be quantitatively obtained as listed in Table 1. The C_m and ΔG_{H_2O} for the BSA with Ca^{2+}, Zn^{2+} and Cd^{2+} ions in the unfolding process are decreased with respect to that of BSA without metal ions. However, it is also found these two quantities

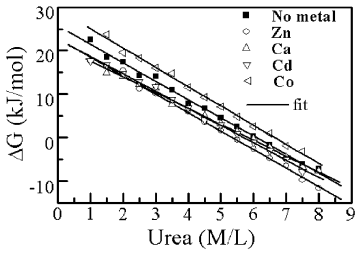

Fig. 5. Free energy change ΔG versus the urea concentration for the unfolding transition of BSA in the presence of metal ions (Zn^{2+}, Ca^{2+}, Cd^{2+} and Co^{2+}) and in the absence of metal ions.

Table 1
The urea-induced unfolding transition parameters induced by fluorescence spectra

Sample	ΔG_{H_2O} /kJ M^{-1}	C_m/M	m/kJ mol^{-1} M^{-1}
Without Metal	26.04	5.88	5.96
Zn^{2+}	22.20	5.52	3.93
Ca^{2+}	21.36	5.84	3.63
Cd^{2+}	22.61	5.61	3.95
Co^{2+}	29.50	6.55	4.49

for BSA with Co^{2+} ions are all increased. Thus, Ca^{2+}, Zn^{2+} and Cd^{2+} ions weaken the conformational stability of BSA, while Co^{2+} ions enhance the conformational stability. The values of m are decreased in the presence of four kinds of metal ions, indicating that the solvent-exposed surface of the BSA molecules in the unfolding process is less than that of BSA without metal ions. This is related to the loss of the solvent-exposed surface due to the coordinative interaction between BSA and metal ions. The values of ΔG_{H_2O} and C_m obtained from the CD measurements also show evidences that Ca^{2+} and Cd^{2+} ions stabilize the secondary structures of BSA in the unfolding process of BSA and Zn^{2+} and Co^{2+} ions destabilize the secondary structures.

4. Conclusions

In summary, the influence of metal ions (Zn^{2+}, Ca^{2+}, Cd^{2+} and Co^{2+}) on the folding pathway and conformational stability of bovine serum albumin (BSA) have been studied. In the presence of Ca^{2+} or Cd^{2+} ions, the unfolding processes of BSA are of two-state behavior, while in the presence of Zn^{2+} or Co^{2+} ions, the unfolding processes of BSA remain of three-state behavior. The construction of ion-binding sites in proteins is important for the design of proteins with high thermo-stability. Therefore, the effects of metal ions on various aspects of proteins determined by the complicated interactions between the metal ions and residues deserve further study.

Acknowledgments

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